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Expression, Reconstitution and Characterization of CDK2/Cyclin A Kinase

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Expression, Reconstitution and Characterization of CDK2/Cyclin A Kinase" submitted by Xujing Zhu in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The progression of the cell division cycle in eucaryotes is regulated by a family of protein serine/threonine kinases called cyclin-dependent kinases (cdks). While considerable progresses have been made towards the elucidation of the functions and regulations of these kinases in cell cycle control, their enzymological characterizations have been limited. This is partly due to the difficulty in obtaining well defined and high quality samples of these kinases. In the present study, I have attempted to use bacterially expressed cdk2 and cyclin A to reconstitute a highly active kinase.

In addition to depending on cyclin, the activities of several cdks have been found to depend on the phosphorylation of a specific threonine residue by a distinct protein kinase, cdk activating kinase (CAK). In the present study, a partially purified preparation of CAK was obtained from bovine brain. Using this preparation, a bacterial expressed cdk2 could be phosphorylated on a threonine residue, and become activated in the presence of a bacterial expressed cyclin A. Optimal conditions for the reconstitution of the active cdk2/cyclin A kinase activity with the brain CAK have been established. A highly activated cdk2/cyclin A kinase with specific histone H1 peptide phosphorylation activity in the range of micromolar phosphate transferred per min. per mg has been isolated. To determine the

phosphorylation site specificity, the phosphorylation of a synthetic peptide P-K-T-P-K-K-A-K-K-L derived from the cdc2 kinase phosphorylation site and a set of substitution analogous on the peptide by the reconstituted cdk2/cyclin A have been characterized kinetically. These peptides have been used previously to elucidate the site specificity of neuronal cdc2-like kinase (nclk). The results indicate that cdk2/cyclin A kinase and nclk have similar phosphorylation site specificity in that both kinases recognize the consensus sequence motif: -S/T-P-X-K/R-. However, some of the secondary positive substrate determinants for nclk, such as the proline at -2 position, the second residue aminoterminal to the phosphorylation site, and lysines at +5 and +6 positions, the fifth and sixth residues carboxylterminal to the phosphorylation site respectively, do not appear to play significant roles in the substrate activity of the peptide for cdk2/cyclin A.

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To Ning and Wei

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INTRODUCTION

1. $p34^{cdc2}$

Genetic analysis of the cell cycle was carried out in both budding yeast and fission yeast by analysis of a collection of genes known as the cell division cycle (cdc) mutations (Hartwell, 1974; Nurse, 1975). In particular, two mutants, cdc2 in *Schizosaccharomyces pombe* and CDC28 in *Saccharomyces cerevisiae*, were identified since they regulated distinct transitions in the cell cycle: passage through Start, in which the cell becomes committed to beginning S phase, and the G2 to M transition (Beach et al., 1982; Nurse et al., 1981). Subsequently, sequencing of both genes revealed that cdc2 and CDC28 encoded highly homologous proteins (Hindley and Phear, 1984; Lorincz and Reed, 1984). Furthermore, a protein that displayed remarkble sequence homology to cdc2/CDC28 has been identified in HeLa cells (Lee and Nurse, 1987). This indicated that the cdc2 gene was highly conserved throughout evolution and the function of this gene in the regulation of the cell cycle was also found to be conserved in all eukaryotes (Nurse, 1990).

To study the regulation of the cell cycle, two approaches have been used. Primarily, genetic mutation has been used to characterize the function of the cdc2 gene in promoting transition across the

G2/M-phase border. cdc2 was found to be required for the normal progression of cells through mitosis. When a cdc2 temperature-sensitive mutant was incubated at the restrictive temperature, cells could be blocked at the G2/M phase border. In contrast, cdc2 Y15A mutant was shown to cause cells to enter mitosis prematurely (Moreno et al., 1989; Draetta and Beach, 1989; Lee and Nurse, 1988).

Independently of genetic studies, biochemical analysis of the cell cycle in Xenopus oocytes identified a component that caused interphase cells to enter mitosis. This protein complex was called maturation promoting factor or MPF. Microinjection of MPF into immature oocytes was able to induce repeated cell cycles (Masui and Markert, 1971; Reynhout and Smith, 1974). The highly purified MPF from Xenopus was shown to contain a 34 kDa subunit which reacted with specific antibodies raised to either the yeast or human $p34^{cdc2}$ protein. This subunit was identified as a Xenopus homologue of $p34^{cdc2}$ (Dunphy at al., 1988; Gautier at al., 1988).

From their chemical structure, $p34^{cdc2}$ proteins have been proposed to be protein kinases: enzymes that transfer phosphate groups from ATP (adenosine triphosphate) to protein. Residues 9-38 of $p34^{cdc2}$ proteins are homologous to the highly conserved ATP-binding site of protein kinases. A mutated $p34^{cdc2}$ with an Arg instead of a Lys at position 33 can not rescue a cdc2 temperature-sensitive strain of *S. pombe*, suggesting that Lys 33 as a part of

ATP-binding site is essential for $p34^{cdc2}$ biological activity (Booher and Beach, 1986). Sebsequently, the proposal that $p34^{cdc2}$ was a protein kinase was verified by *in vitro* assays of protein kinase activity of the immunopricipates of the protein using histone H1 as a substrate. This protein kinase was found to be transiently activated as cells entered mitosis and inactivated as cells exited mitosis (Norbury and Nurse, 1990).

As a protein kinase, the function of $p34^{cdc2}$ is critical for G1/S and G2/M transitions during the cell cycle. The discovery of the functional $p34^{cdc2}$ homologues from a wide variety of species including humans indicates that $p34^{cdc2}$ is a universal cell cycle regulator (Nurse, 1990).

2. Regulation of $p34^{cdc2}$ by Cyclins

During cell cycle progression the concentration of $p34^{cdc2}$ remains relatively constant although its protein kinase activity changes dramatically (Simanis and Nurse, 1986; Draetta et al., 1989). The cell cycle-dependent kinase activity is regulated in part by the association of $p34^{cdc2}$ with a class of regulatory protein named cyclins (Pines and Hunter, 1991a; Pines and Hunter, 1991b).

Cyclins were first identified as proteins in eggs of sea urchins and clams. In these cells the abundance of cyclins oscillated with

progression through the embryonic cell cycle, being high in mitosis, and abruptly low in interphase (Evans at al., 1983; Swenson et al., 1986). The regulatory function of cyclins was first substantiated by showing that microinjection of cyclin mRNAs into Xenopus oocytes, or into cell-free Xenopus egg extracts, could induce maturation (Pines and Hunt, 1987; Swenson and Ruderman, 1986). Subsequently, a complementary experiment showed that preventing translation of endogenous cyclin B mRNAs blocked the ability of Xenopus egg extracts to enter mitosis (Minshull et al., 1989). These results showed that cyclin was required for the maturation of Xenopus oocytes.

According to their distinctive cyclic pattern of accumulation and degradation during cell division cycle, two subfamilies of cyclins, cyclin A and B, were designated (Swenson and Ruderman, 1986). The $p34^{cdc2}$/cyclin B complex is the best characterized form of $p34^{cdc2}$ kinase which displays histone H1 kinase activity; most studies have found that the uncomplexed free $p34^{cdc2}$ subunit does not phosphorylate histone (Brizuela et al., 1989). Cyclin B synthesis and degradation are both necessary and sufficient to maintain the alteration between S and M phases in a cell-free extract prepared from early Xenopus embryos (Murray and Kirschner, 1989a). As in embryonic systems, the quantity of cyclin B in somatic cells is low after mitosis, and gradually accumulates during interphase until the maximum level is reached in late G2 phase, when $p34^{cdc2}$/cyclin B is

activated. After induction of M phase, cyclin B is rapidly proteolyzed, and the active $p34^{cdc2}$/cyclin B complex is disintegrated (Murray and Kischner, 1989b).

Cyclin A associates with $p33^{cdk2}$ (Elledge et al., 1992; Rosenblatt et al., 1992; Tsai et al., 1991), a $p34^{cdc2}$ homologue, during S phase and G2 phase but also associates with $p34^{cdc2}$ (Clarke et al., 1992, Koff et al., 1992). Like cyclin B, the level of cyclin A expression in HeLa cells is cell cycle dependent, but its activity appears just before cyclin B (Pines and Hunter, 1990b). Cyclin A-associated $p34^{cdc2}$ kinase was found more active in cells blocked in S phase than cells blocked in mitosis, suggesting a function in regulation of the cells entry into S phase (Giordano et al., 1989). *In vitro* reconstitution of $p34^{cdc2}$/cyclin A and $p33^{cdk2}$/cyclin A kinase activity showed that cyclin A is required for the activation of both $p34^{cdc2}$ and $p33^{cdk2}$ as kinases (Poon et al., 1993; Fesquet et al., 1993; Fisher et al., 1994).

Additional cyclins have since been identified in animal cells and the protein family now comprises of at least eight subtypes, designated cyclin A to H.

3. Multiple Forms of cyclin/cdk kinase

The cdc2-related proteins have grown as a superfamily based on sequence homology screening and functional complementation. In addition to yeast cdc2 homologues, in mammalian cells, cdk1 (cdc2), cdk2, 3, 4, 5, 6, and 7 have been characterized. Most of cdks associate with cyclin-like proteins, except cdk3 and cdk5, and function in cell cycle regulation. cdk3 has never been found in association with cyclins although it can complement CDC28 mutants of *S. cerevisiae* (Meyerson et al., 1992; van den Heuvel et al., 1993). cdk5 kinase was identified both by being purified from bovine brain as the catalytic subunit of a novel protein kinase and as a novel clone by screening a human cDNA library based on its close primary sequence homology to the human cdc2 kinase (Lew et al, 1992; Meyerson et al., 1992). cdk5 was found to associate with a specific activator, p35, which revealed very weak homology to cyclins. However, much like other cyclin/cdk complexes, cdk5 activation requires binding of p35. (Lew et al., 1992; Lew et al., 1994; Tsai et al., 1994).

In addition to the existence of cyclin A and cyclin B, there has been a recent population explosion in the cyclin superfamily. Newly identified members of this family are defined as cyclin-like based on sequence homology within a narrow region referred to as the "cyclinbox" (Lees and Harlow, 1993). In many cases the sequence conservation is extremely weak, displaying only a few amino acids which correspond to conserved residues in cyclins A or B. Thus,

apart from sequence similarity, another feature that can be used to classify a protein as a cyclin is its association with and activation of a cdk protein kinase during the cell cycle (Xiong and Beach, 1991).

Selected members of the cyclin family associate with members the of cdc2 family to form multiple active kinases which are involved in the cell cycle regulation. For example, G1 cyclins in budding yeast, CLN1, 2 and 3, could rescue CDC28 temperature sensitive mutants and to confer resistance to cell arrest by pheromone action (Cross, 1988; Nash et al., 1988; Hadwiger et al., 1989; Reed, 1991). CLN cyclins accumulate and peak in G1 phase and associate with CDC28 which is required for cells to enter S phase (Reed, 1991). Overexpression or deletion mutation of CLN cyclins caused cells to either advance to S phase or arrest in G1 phase (Tyers, et al., 1993;).

This pattern of multiple cyclins associating with a cdk was also observed in mammalian cells. In mammalian cells, key regulators of G1 progression include three D-type cyclins (D1, D2 and D3), cyclin C and cyclin E (Lew et al., 1991; Matusushime et al., 1991). D-type cyclins associate with and activate cdk4 or cdk6 (Meyerson and Harlow, 1994; Tam et al., 1994; Bates et al., 1994a; Bates et al., 1994b). Overexpression of cyclin D1, D2 or D3 shortens the G1 phase of the cell cycle in various cell types, while deletion of cyclin D1 blocks the progression of human fibroblasts through G1 (Ando et al.,

1993; Baldin et al., 1993; Kato et al., 1993a; Quelle et al., 1993). cdk4 is activated by D-type cyclins and the complexes can phosphorylate pRb, the product of the retinoblastoma gene, to function as a positive regulator of G1 progression (Matusushime et al., 1992; Matusushime et al., 1994; Kato et al., 1993b; Ewen et al., 1993; Tam et al., 1994). cdk6 is associated with cyclin D1, D2 and D3 in lysates of human cells and can be activated by coexpression with D-type cyclins in Sf9 insect cells. The activation of cdk6 kinase occurs during mid-G1, prior to the activation of cdk2 kinase, suggesting that cdk6 could link growth factor stimulation with the onset of cell cycle progression (Meyerson and Harlow, 1994).

Unlike the D-type cyclins, cyclin E combines with cdk2 and induces maximal levels of cyclin E/cdk2 kinase acitvity shortly before S phase entry (Dulic et al., 1992; Koff et al., 1992; Wimmel et al., 1994). Human cyclin E/cdk2 can phosphorylate histone H1 and pRB *in vitro* and rescue a triple CLN mutation in *S. cerevisiae* (Sewing et al., 1994).

In addition to G1 cyclins in budding yeast and mammalian cells, a group of G2 cyclins, CLB1, 2, 3, 4, 5, and 6 show strong homology with cyclin B (Ghiara et al., 1991; Surana et al., 1991; Epstein and Cross, 1992; Schwob and Nasmyth, 1993). In budding yeast CLB1, 2, 3 and 4 associate with CDC28 which are necessary for the formation and function of the mitotic apparatus. Deletion or overexpression of

all four genes is lethal and causes cells to arrest in either G2 or mitosis (Fitch et al., 1992; Ghiara et al., 1991). CLB5 and CLB6 associate with CDC28 which are also involved in S phase entry (Epstein and Cross, 1992; Schwob et al., 1994).

The existence of several classes of cyclins and mutiple cdc2-related protein kinases has changed the view of the cell cycle as a cdc2 cycle (Nurse, 1990). Various combinations of cyclin/cdk kinase indicate that the cell cycle control is excercised at various execution points by distinct cyclin-dependent kinases. Given how many signals act upon the cell cycle, it is not surprising that such complexity is reflected in the number of cyclin/cdk complexes, each likely to have distinct biological properties.

4. Regulation of $p34^{cdc2}$ by Phosphorylation

Although it is clear that cyclins are regulatory proteins, their patterns of synthesis and destruction do not fully account for $p34^{cdc2}$ kinase activity. For example, during G2, cyclin B levels were already high, yet $p34^{cdc2}$ kinase activity was low. Thus the activity of $p34^{cdc2}$ kinase required not only association with cyclins, but also some other mechanism, possibly phosphorylation/dephosphorylation reactions. This was shown to be the case in fission yeast (Gould et al., 1991; Gould and Nurse, 1989). Using highly synchronized cultures, it was shown that dephosphorylation of tyrosine residues

occurred as *S. pombe* cells enter mitosis. Subsequently the tyrosine phosphorylation in $p34^{cdc2}$ was localized to amino acid 15 by tryptic phosphopeptide and site-directed mutagenesis analysis. Comparison of the phosphoamino acid in phosphopeptide derived from wild-type *S. pombe* $p34^{cdc2}$ *in vitro* and the phosphoamino acid of $p34^{cdc2}$ in both *S. pombe* and murine in vivo revealed that only Tyr 15 could be phosphorylated *in vivo.* This result was also confirmed by substitution of Tyr 15 with phenylalanine which resulted in the absence of phosphotyrosine in $p34^{cdc2}$. Furthermore, expression of cdc2 Phe 15 mutant advanced yeast cells prematurely into mitosis. These results demonstrated that tyrosine phosphorylation/dephosphorylation directly regulated $p34^{cdc2}$ function and thereby regulated entry of the cell into mitosis (Gould and Nurse, 1989).

The conservation of regulatory phosphorylation of $p34^{cdc2}$ was later confirmed in HeLa cells by mutation of Tyr 15 to phenylalanine. As expected, HeLa cells expressing the mutant protein underwent premature mitosis (Krek and Nigg, 1991a). Biochemical studies have also confirmed that dephosphorylation of Tyr 15 with phosphatases *in vitro* stimulates the kinase activity of $p34^{cdc2}$ (Gould et al., 1990; Pondaven et al., 1990). In contrast, agents such as vanadate prevented dephosphorylation of $p34^{cdc2}$ *in vivo* and consequently prevented cells from entering mitosis (Morla et al., 1989). Therefore, phosphorylation of Tyr 15 is associated with an

inhibition of kinase activity and entry into mitosis parallels dephosphorylation of $p34^{cdc2}$ on Tyr 15 (Draetta et al., 1988; Gould and Nurse, 1989).

The main protein kinase responsible for inhibitory phosphorylation of $p34^{cdc2}$ on Tyr 15 is wee1, the product of a conserved gene first discovered in fission yeast as negatively controlling the G2 to M phase transition (Russell and Nurse, 1987; Featherstone and Russell, 1991; Parker et al., 1991 and Parker et al., 1992). Overproduction of $p34^{cdc2}$, cyclins (A and B) and the fission yeast wee1 gene product (p107wee1) using a baculoviral expression system showed that phosphorylation of $p34^{cdc2}$ on tyrosine was absolutely dependent upon the presence of catalytically active $p107^{wee1}$ (Parker et al., 1991). Also *in vitro*, the expressed human wee1 has been shown to phosphorylate a recombinant $p34^{cdc2}$/cyclin B complex at Tyr 15, resulting in inhibition of histone kinase activity (Parker and Piwnica-Worms, 1992; Honda et al., 1992).

In higher eukaryotes, an additional phosphorylaton on threonine also occures. The dephosphorylation of threonine 14 acts conjunction with that of Tyr 15 to activate $p34^{cdc2}$ kinase (Krek and Nigg, 1991b; Norbury et al., 1991). The Thr 14 kinase has not yet been found. The control of cell cycle transitions involve both a negative inhibitory pathway, including the wee1 gene product, and a positive activating pathway, including CDC25 gene product. The balance of these two

pathways regulates $p34^{cdc2}$ function and advances or delays the onset of mitosis. Dephosphorylation of Tyr 15 appears to be under the control of the $p80^{CDC25}$ phosphatase, the product of a gene first discovered as positively controlling the G2 to M phase transition in fission yeast (Russell and Nurse, 1986). In cell-free system, the recombinant $p80^{CDC25}$ protein could dephosphorylate immunoprecipitated $p34^{cdc2}$ on Tyr 15 and activate $p34^{cdc2}$/cyclin kinase (Kumagai and Dunphy, 1991). This result was confirmed by using highly purified phosphorylated $p34^{cdc2}$/cyclin complexes (Stransfeld et al., 1991). Subsequently, CDC25 was found to be a dual function phosphatase which may dephosphorylate both Thr 14 and Tyr 15 of $p34^{cdc2}$ (Millar et al., 1991; Guan et al., 1991).

A third specific residue, threonine 161 in higher eukaryotes (residue 167 in yeast), has been shown to be absolutely required for $p34^{cdc2}$ activation by phosphorylation (Gould et al., 1991; Krek and Nigg, 1991b; Gu at al., 1992). Recently, the kinase responsible for Thr 161 phosphorylation has been identified and purified. It was termed CAK, cdk-activating kinase (Solomon et al., 1993; Poon et al., 1993; Fesquet et al., 1993; Fisher et al., 1994; Labbé et al., 1994 and Tassan et al., 1994). Studies of CAK have shown that it can phosphorylate and activate complexes of $p34^{cdc2}$/cyclin A and $p34^{cdc2}$/cyclin B on Thr 161 or $p33^{cdk2}$/cyclin A on Thr 160. Tryptic phosphopeptide mapping and phosphoamino acid analysis showed that $p34^{cdc2}$ and $p33^{cdk2}$ were phosphorylated exclusively on threonine in

a single tryptic peptide (Tassen et al., 1994; Poon at al., 1993; Fesquet et al., 1993). Furthermore, substitution of Thr 161 of $p34^{cdc2}$ or Thr 160 of $p33^{cdk2}$ with a non-phosphorylatable residue, alanine, abolished phosphorylation of cdc2 and cdk2 by CAK. Concomitantly, either T161A mutant of $p34^{cdc2}$ or T160A mutant of $p33^{cdk2}$ could not be activated by cyclins as a protein kinase (Poon et al., 1993; Fesquet et al., 1993). CAK together with wee1 and CDC25 are involved in a regulatory network during the cell cycle. Understanding this network is essential to understanding the mechanisms of the full cell cycle.

All the above evidence builds up a current model which indicates the logic of the phosphorylation regulation of $p34^{cdc2}$: the only significantly active forms of the kinase have phosphorylated Thr161 and nonphosphorylated Thr 14 and Tyr 15 residues. This active kinase is required for progression at the point of Start in G1 phase and on to M phase during the cell cycle.

5. CAK

An enzymatic activity has been identified in Xenopus egg extracts and in mammalian tissue culture cell extracts that phosphorylates $p34^{cdc2}$ at Thr 161 in the presence of cyclin (Solomon et al., 1992; Desai et al., 1992). This enzyme was termed CAK, for cdk-activating kinase. Phosphorylation of an apparently

catalytically inactive form of Xenopus $p34^{cdc2}$ by partially purified CAK suggested that CAK was a protein kinase. The CAK-induced phosphorylation of $p34^{cdc2}$ was required for $p34^{cdc2}$ protein kinase activity, but not for its association with cyclin (Solomon et al., 1992). By immunoblotting, immunoprecipitation and immunodepletion of CAK from Xenopus egg extracts, a $p40^{MO15}$-like protein was found as a subunit of CAK (Solomon et al., 1993; Poon et al., 1993; Fesquet et al., 1993).

MO15 was originally cloned in a search for Xenopus $p34^{cdc2}$-related protein by using oligonucleotides deduced from the conserved sequence of protein kinase catalytic domains (Shuttleworth et al., 1990). *In vitro* experiments showed that bacterially expressed $p40^{MO15}$ could phosphorylate and activate $p34^{cdc2}$/cyclin A on Thr 161 and $p33^{cdk2}$/cyclin A on Thr 160 after preincubation of $p40^{MO15}$ in either Xenopus egg or HeLa cell extracts. CAK purified from Xenopus egg, starfish oocytes and Hela cells contains $p40^{MO15}$ or its homologues as the major subunit, demonstrating that MO15 is a gene conserved throughout evolution (Solomon et al., 1993; Fesquet et al., 1993; Fisher et al., 1994; Tassan et al., 1994).

Wild-type $p40^{MO15}$ protein expressed in bacteria does not possess kinase activity, but acquires $p34^{cdc2}$-T161 and $p33^{cdk2}$-T160 kinase activity after incubation with cell extract and ATP.

This indicates that $p40^{MO15}$ requires activation by phosphorylation or association with some other companion subunits. Further purification of CAK showed that a protein subunit of similar molecular weight (36 kDa in Xenopus egg; 37 kDa in HeLa cells; and 40 kDa in starfish oocytes), associates with MO15 (Labbé at al., 1994; Fisher et al., 1994; Mäkelä et al., 1994; Fesquet et al., 1993). On the basis of sequence homologies of human 37 kDa subunit, this subunit was identified as a novel member of the cyclin family, cyclin H (Fisher et al., 1994). Yeast two hybrid screen showed that human cyclin H is a MO15-associated protein; reconstitution of human CAK activity required association of MO15 with cyclin H (Mäkelä et al., 1994; Fisher et al., 1994). Since the activity of MO15, a CDK-like protein, is dependent on cyclin H, MO15 is a cyclin-dependent kinase, so called CDK7 (Fisher et al., 1994). Another MO15 associated protien, a 32 kDa protein, was also purified from HeLa cells but its sequence and function are still unknown (Tassan et al., 1994).

Following CAK purification, the regulation of CAK has been investigated. There is no substantial variation on the level or kinase activity of $p40^{MO15}$ during progress through either embryonic or somatic cell cycles. However, bacterially expressed $p40^{MO15}$ was more strongly activated by M-phase frog egg extract than by interphase egg extracts (Poon et al., 1994; Tassan et al., 1994). Recently, $p40^{MO15}$ was found to be phosphorylated on serine 170 and

threonine 176 using proteolytic degradation, radiosequencing of tryptic peptides generated from $p40^{MO15}$ and analysis mutants prepared by site-directed mutagenesis. Substitution of Thr 176 with alanine results in a T176A mutant of $p40^{MO15}$ that can not be phosphorylated at this site. This mutant is inactive. In contrast, phosphorylation of Ser 170 is dispensable although *in vitro* Ser 170 can be phosphorylated by active $p34^{cdc2}/p33^{cdk2}$ (Labbé et al., 1994; Poon et al., 1994). However the kinase responsible for the phosphorylation of MO15 on Thr 176 and Ser 170 has not been discovered and also the role of Ser 170 phosphorylation remains unknown.

$p40^{MO15}$ as a cdc2 related protein kinase contains Thr 176 at the equivalent position of Thr 161 in cdc2 (Thr 160 in cdk2). Phosphorylation of Thr 176 is required for activation of CAK, inplicating a positive regulation pathway upstream of CAK. Besides, association of cyclin H with $p40^{MO15}$ could reconstitute CAK activity *in vitro*, indicating that $p40^{MO15}$ may have the same cyclin regulatory mechanism as that of cdc2 kinase and that the cyclin regulation of cdc2 may be universal.

In spite of the sequence similarity between $p40^{MO15}$ and $p34^{cdc2}$, CAK appears to have a different and very limited substrate specificity. CAK has no activity towards conventional $p34^{cdc2}$ substrates such as histone H1 (Solomon et al., 1992). Also, no

peptide substrates have been found for CAK. Except cdc2 and cdk2, it is still unclear how many more cdks CAK can phosphorylate. Further investigation of the regulatory mechanism of CAK and the search of some other substrates of CAK will lead to a better understanding of signal transduction parthways and the regulatory checkpoints of the cell cycle.

6. Inhibitors

The discovery of genes encoding cdk inhibitors has had a major impact on our understanding of the fundamental mechanism underlying negative growth control and checkpoints. The universality of negative cell cycle control via cdk inhibitors is indicated by the identification of inhibitors in budding and fission yeast, as well as in mammalian cells.

The first cdk inhibitory factor, a 40 kDa protein (p40) was detected in budding yeast as a protein associated with CDC28 (Mendenhall et al., 1987; Mendenhall, 1993). The gene encoding this protein was cloned and called Sic1 (Nugroho et al., 1994). Sic1 was shown to inhibit the histone H1 kinase activity of immunoprecipitated or purified CDC28-cyclin complexes. Overproduction of Sic1 in cells produced a phenotype similar to that of cells lacking mitotic cyclins, suggesting a role in inhibiting mitosis. Cells lacking Sic1 were viable but contained broken or

damaged chromosomes, suggesting a problem in DNA replication in S phase (Nugroho et al., 1994).

Also in budding yeast, a 30 kDa protein, Far1, was found to bind to CDC28 and inhibit histone H1 kinase activity of CDC28 both *in vivo* and *in vitro* (Chang et al., 1990; Chang et al., 1992; Peter et al., 1993). Far1 shows some specificity for CLN1-CDC28 and CLN2-CDC28 complexes whereas it is unable to inhibit CLB2 or CLB5 kinase complexes (Peter et al., 1994). *In vivo*, the stability of Far1 is cell cycle regulated, such that it is only present in G1 phase and degrades just after G1/S transition (Mckinney et al., 1993).

The rum1 gene was identified by a screen for cDNAs from *S. pombe* that are lethal when expressed at high levels (Moreno and Nurse, 1994). When overproduced rum1 caused multiple rounds of DNA synthesis in the absence of mitosis, causing cells to have n_x more DNA. Rum1 was found to inhibit cdc2 kinase activity *in vitro* (Elledge and Harper, 1994).

In mammalian cells a 21 kDa protein, p21, was first identified in anti-cyclin D1 immunoprecipitates of human diploid fibroblasts as a component of cyclin D complexes which also contain cdks and proliferating cell nuclear antigen (PCNA) (Xiong et al., 1992). After microsequncing the purified p21 protein, the p21 gene was isolated from a human cDNA library (Xiong et al., 1993a). In other

laboratories, p21 was also isolated through several other approaches: (i) as a gene whose transcription is induced by p53 function, WAF1 (El-Deiry et al., 1993); (ii) in a search for cdk2 regulators by the two-hybrid screening technique, Cip1 (Harper et al., 1993); and (iii) in a search for cDNAs corresponding to mRNAs preferentially expressed in human cells that can block cells from entering S phase when overexpressed by transient transfection, Sdi1 (Noda et al., 1994). p21 was recently found to associate with and inhibit the kinase activity of a variety of cyclin-cdk complexes produced as either purified proteins from bacteria, immunoprecipitates from mammalian cells, or in crude lysate from recombinant baculovirus infected insect cells. These cdk complexes include cyclin A-cdc2, cyclin B-cdc2, cyclin A-cdk2, cyclin E-cdk2, cyclin D1-cdk4, and cyclin D2-cdk4 (Harper et al., 1993; Xiong et al., 1993a; Gu et al., 1993; Zhang et al., 1993; Li et al., 1994). Furthermore, p21 inhibits kinase activity toward all substrates tested, including histone H1 and pRb. The fact that p21 does not require the presence of additional cellular proteins to perform its inhibitory function was demonstrated by its ability to inhibit the bacterially produced cyclin A-cdk2 complex (Harper et al., 1993; Gu et al., 1993; Xiong et al., 1993a).

Further examination of cyclin-cdk complexes in normal and in transformed cells revealed two more small cyclin-cdk associated proteins. Firstly, p16 is a protein that was stimulated to

specifically form a complex with cdk4 in transformed cells lacking pRb function and to inhibit cyclin D-cdk4 complexes by blocking and/or dissociating the D-type cyclin association (Serrano et al., 1993; Xiong et al., 1993b). Secondly, p19 is a protein present in cyclin A-cdk complexes in transformed cells; however, the molecular identity of p19 is still under investigation (Xiong et al., 1993b).

In mink lung fibroblasts, G1 phase arrest caused by treatment with transforming growth factor (TGF)-ß or accumulation of an inactive form of cdk2-cyclin E containing a 27 kDa inhibitor (Koff et al., 1993; Polyak et al., 1994a). The cDNA for p27 was cloned independently using protein sequence information and by the two hybrid system with CDK4-cyclin D as the target (Polyak et al., 1994b; Totoshima and Hunter, 1994). p27 was shown to have sequence similarity to p21 and *in vitro*, p27 fusion protein inhibited the same spectrum of cdks as p21. Association of p27 with cdk2-cyclin E can also block phosphorylation of cdk2 on Thr 160 by CAK (Polyak et al., 1994b). Recombinant p27 is a potent inhibitor of cyclin D-cdk4 and cyclin A-cdk2 and a weaker inhibitor of cyclin B-cdc2 (Totoshima and Hunter, 1994).

Cdi, a human 21 kDa protein was isolated in a yeast genetic selection for interacting proteins by virtue of its ability to interact with a number of cdks including cdc2, cdk2 and cdk3 (Gyuris et al.,

1993; Hannon et al., 1994). Overexpression of Cdi delays cell cycle progression (Gyuris et al., 1993). Finally, a 28 kDa protein, p28, was found to associate with cdk6 in human T lymphocytes stimulated by phytohemagglutinin; however its identity is unknown (Meyerson and Harlow, 1994).

The multiple types of cdk inhibitors are the natural cell cycle brakes which serve unique functions in controlling cell cycle arrest in response to negative growth stimuli. They are the results of versatile messengers of growth arrest signals that are involved in a variety of regulatory networks. Elucidation of mechanisms for negative regulation of the cell cycle will contribute to the understanding of the complex control of cell cycle transition and cell proliferation, as well as the process of cellular transformation.

7. Substrate Sequence Specificity

While yeast genetic and cell biological studies have revealed highly complex regulation of $p34^{cdc2}$ kinase and related kinases, few enzymological characterizations of these enzymes have been carried out. One important consideration of any protein kinase is its sequence specificity. The three dimensional structure of protein kinases enable them to recognize sequence elements immediately surrounding the phosphorylation site that are considered essential for phosphorylation by the kinase. This is referred to as a consensus

sequence (Kennelly and Krebs, 1991). The first substrate regulation sequence for the cdc2 kinase was identified in histone H1 which is an excellent substrate for the enzyme *in vitro* and *in vivo* (Langan et al., 1980). The recognition sequence for $p34^{cdc2}$ kinase phosphorylation has been proposed to be S/T-P-X-K/R, based on the analysis of *in vitro* and *in vivo* phosphorylation sites of numerous $p34^{cdc2}$ substrates (Pines and Hunter, 1990; Draetta, 1990; Maller, 1990; Peter et al., 1990). This sequence was found as the consensus motif in which the phosphorylated residue is followed by a proline residue and X is a polar amino acid (Langan et al., 1989; Vulleit et al., 1989; Moreno and Nurse, 1990). In the case of histone H1, because proline is known to exert structural effects similar to a bend or kink in protein molecules, this motif is predicted to form a ß-turn conformation which is a DNA binding motif (Moreno and Nurse, 1990; Suzuki, 1989). Phosphorylation of this site would disrupt the structure and prevent DNA binding. The subsequent release of histone H1 may contribute to chromosome condensation at mitosis (Pines and Hunter, 1990; Moreno and Nurse, 1990; Suzuki, 1989; Churchill and Suzuki, 1989).

More recently, this proposed substrate consensus motif was verified by using synthetic peptides to systematically investigate the phosphorylation specificity of the bovine brain cdc2-like kinase, nclk, and HeLa cell $p34^{cdc2}$ by challenging the kinase with peptides of different sequence (Beaudette et al., 1993). The peptide P-K-T-P-

K-K-A-K-K-L derived from the *in vitro* $p34^{cdc2}$ phosphorylation site of histone H1 was found to be an excellent substrate for both $p34^{cdc2}$ and the bovine nclk. Tests with other substrates revealed that these two kinases displayed similar substrate specificities. In addition to the absolute requirement for a proline residue immediately COOH-terminal to the phosphorylatable residue (+1) and a basic residue at the +3 position, a basic amino acid at the +2 position was greatly preferred over an acidic amino acid. A proline residue at the -2 position and a cluster of basic amino acids further COOH-terminal to the consensus motif were also found to be important for substrate binding (Beaudette et al., 1993).

Although this motif is widely accepted as the consensus sequence for $p34^{cdc2}$ kinase, there is an evidence to suggest that various forms of $p34^{cdc2}$/cyclin have slightly different substrate specificities. Cyclin A/$p34^{cdc2}$ immunoprecipates from Xenopus egg extracts was shown to phosphorylate sea urchin sperm histone H2B more strongly than histone H1 whereas cyclin B/$p34^{cdc2}$ immunoprecipates phosphorylated histone H1 at about twice the rate of histone H2B. However, cyclin A/$p34^{cdc2}$ immunoprecipates were found as efficient at phosphorylating histone H1 as cyclin B/p34cdc2 (Minshull et al., 1990).

With the existence of multiple forms of cdc2 kinases and cyclins, there is also evidence to suggest that different cdks binding to

different partners may have an altered substrate specificities. Substrate assay of cdk2 and human $p34^{cdc2}$ showed that both formed complexes with cyclin A and phosphorylated histone H1 equally well but cyclin A/$p34^{cdc2}$ complex could phosphorylate another *in vitro* substrate, RF-A, 10 fold better than cyclin A/cdk2 complex (Elledge et al., 1992). In addition, cyclin D/cdk4 and cyclin D/cdk6 do not have any histone H1 kinase activity, yet both phosphorylate the retinoblastoma gene product, pRb (Matusushime et al., 1992; Ewen et al., 1993; Tam et al., 1994; Meyerson and Harlow, 1994). Human cyclin E/cdk2 was shown to phosphorylate both histone H1 and pRb *in vitro* (Sewing et al., 1994). Thus the cyclin partner may influence the substrate specificity of the cdk kinase subunit. But one should also recall that the cyclin subunit may direct the kinase to unique substrates, thus altering its kinetics.

The newly resolved crystal structures of cdk2 and cAMP dependent kinase provide unprecedented insight into the precise atomic movements underlying protein kinase regulation (De Bondt et al., 1993; Taylor et al., 1993). The crystal structure analysis has showed us how cdk2 kinase interacts with a substrate and why monomeric cdk2 is inactive. Firstly, although the monomeric cdk2 can bind ATP it can not cleave it , nor can it transfer the γ phosphate to a substrate because in the cdk2-ATP structure, the phosphate β-γ bond of ATP is almost perpendicular to the hydroxyl group of the substrate serine/threonine. As a result, the in-line phosphotransfer

reaction is not possible in cdk2 (De Bondt et al., 1993). Secondly, the substrate-binding site of cdk2 is blocked by an alignment of the DFG-APE loop, ∂L12 helix. This 'T loop' would prevent any substrate from interacting with the bound ATP. Of particular importance, this loop contains the residue Thr 160, the site of phosphorylation by CAK. Both cyclin binding and Thr 160 phosphorylation result in movement of the T loop away from the active site and allow substrates to bind to cdk2 kinase (De Bondt et al., 1993; Pines, 1993; Morgan and De Bondt, 1994). This data give a detailed molecular explanation of why phosphorylation of cdk at this site is likely to be essential for full cdk activity.

Studies of cdk family kinase substrates and substrate specificities will provide more precise molecular explanations as to how the events of S phase and mitosis are brought about during the cell division cycle.

The investigation of the substrate specificities of $p34^{cdc2}$ in HeLa cells and nclk in bovine brain have been carried out in our lab by using synthetic peptides. However, the substrate specificity of cdk2 kinase remains unknown. This thesis is the first attempt to describe the activation of bacterially expressed cdk2/cyclin A complex by partially purified CAK from bovine brain and the characterization of the active cdk2/cyclin A kinase (free of

contamination) in terms of its catalytic and regulatory properties, including a detailed study of its phosphorylation site specificity.

MATERIALS AND METHODS

Materials

DEAE Sepharose CL6B was obtained from Pharmacia Fine Chemicals. Heparin- Sepharose and Mono Q from Pharmacia. Ni-NTA-Agarose was from Qiagen. Hydroxylapatite, molecular weight markers, horseradish peroxidase-linked goat anti-rabbit IgG, nitrocellulose membrane, and Affigel 10 were purchased from Bio-Rad. Anti-human cyclin A mouse monoclonal antibody was from Upstate Biotechnology Inc. [γ-^{32}P]-ATP (4500 Ci/mmol) was obtained from ICN. P81 phosphocellulose was purchased from Whatman. H1 histone was from Boehringer Mannheim. Isopropyl-1-thio-ß-D-galactopyranoside was purchased from Gibco BRL. Microcrystalline cellulose Macherey-Nagel TLC plates were from Alltech. Leupeptin, aprotinin and pepstatin A were purchased from United States Biochemical Corporation. HEPES, SDS and Tris were obtained from Boehringer Mannheim. Methanol was purchased from BDH Inc. All other chemicals were purchased from Sigma.

Assay for cdk2 Kinase Activity

cdk2 and cdk5 protein kinase activity was determined by measuring the incorporation of PO_4^{-2} from [γ-^{32}P]-ATP into histone

H1 (1-18) peptide. Routine assays were carried out at 30°C in a total volume of 30µl containing: 20 mM MOPS pH 7.2, 10 mM $MgCl_2$, 50 mM NaF , 167 µM [γ-^{32}P]-ATP (500-1000 dpm/pmol), 100 µM histone H1 (1-18) peptide, and an aliquot of enzyme sample. After 30 min, the reaction was terminated by 17% HAC and an aliquot was spotted onto 2.5 x 2.5 cm squares of phosphocellulose paper. The paper were washed 4 x 10 min in 0.3% H_3PO_4, once in acetone, and then dried. Radiolabel incorporated into histone H1 (1-18) peptide was quantitated by liquid scintillation counting using a Beckman LKB 1215 scintillation counter.

Determination of Kinetic Constants

Kinetic constants were determined from Lineweaver-Burk plots using linear regression analysis. Each Km and Vmax value was determined at least twice with a minimum of 8 different peptide concentrations per determination. Km values are reported as the mean values.

Protein Concentration Determination

Protein concentration was estimated either by absorbance at 280 nm or by absorbance at 595 nm with Coomassie blue (Bradford, 1976).

Gel Electrophoresis and Autoradiography

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (Laemmli, 1970), and the gel was stained with either Coomassie blue or silver (Wray et al., 1981). Autoradiography was performed at -70°C using Kodak AR-5 film in a Kodak X-OMAT cassette with intensifying screens.

Phosphoamino Acid Analysis and Western Blotting

Phosphoamino acid analysis was performed according to the method of Hunter and Sefton (Hunter and Sefton, 1980). Western blotting was performed according to the method of Towbin (Towbin et al., 1979) using alkaline phosphatase-linked second antibody and p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as the color substrate.

Preparation of cdk2 C-terminal, GST and GST-MO15 Polyclonal Antibodies

A purified synthetic peptide derived from mammalian $p33^{cdk2}$ C-terminal, QDCTKPVPHLRL, was coupled to keyhole limpet hemocyanin via an additional cysteine residue attached to the C-terminus of the peptide. Bacterially expressed GST and GST-MO15 proteins were purified from GSH-Sepharose. Rabbits were immunized according to standard methods and serum was collected (Harlow and Lane, 1988).

After a 50% ammonium sulphate precipitation, antibodies were purified on an Affigel 10 column coupled with the appropriate peptide according to standard protocols (Harlow and Lane, 1988).

Purification of Bacterially Expressed GST-cdk2

GST-cdk2 was transformed into *E. Coli* strain BL21 (DE3) and 1 L cultures were grown to an A_{600} of 0.5-1.0 at 37°C. Synthesis of recombinant proteins was induced with 0.5 μM isopropyl-ß-D galactoside (IPTG) at room temperature overnight. The cells were harvested and washed with PBS (170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). After resuspended in PBS with 1 mM DTT, 0.25 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine and 10 μg/ml trypsin inhibitor, the cells were lysed by French Press. The lysate was centrifuged at 18,000 rpm at 4°C for 20 min and then the supernatant was applied onto a 1 ml column of GSH-Agarose which was equilibrated with PBS. After loading, the column was washed with 10 bed volumer of PBS, PBS with 0.35 M NaCl and PBS, containing 1 mM DTT, 0.25 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine and 10 μg/ml trypsin inhibitor. GST-cdk2 was eluted with 20 mM reduced glutathione in 50 mM Tris-HCl pH 8.0, 1mM DTT. Purified proteins were dialyzed against 20 mM HEPES containing 1 mM DTT, 0.25 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine and 10 μg/ml trypsin inhibitor.

Purification of PA-cyclin A

His-tagged PA-cyclin A was purified by the procedures described by Poon (1993). Overexpression and cell lysis followed the same procedure as described above in "GST-cdk2 purification method". The supernatant was applied onto a Ni-NTA Agarose column. After sample loading, the column was washed with 5 bed volumes of 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole, followed by 5 bed volumes of 20 mM Tris-HCl pH 8.0, 1.0 M NaCl, 25 mM imidazole, 0.5% Tween-20, 0.5% Triton X-100, and finally 5 bed volumes of 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 50 mM imidazole. Histidine-tagged proteins were eluted with 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 150 mM imidazole. Purified proteins were dialyzed against 20 mM Tris-HCl pH 8.0, 20 mM NaCl, 0.5 mM EDTA, containing 1 mM DTT, 0.25 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine and 10 μg/ml trypsin inhibitor.

CAK Activity Assay

Samples to be assayed for their CAK activity were mixed with 0.13 μg GST-cdk2 and 0.24 μg PA-cyclin A, made to 20 mM $MgCl_2$, 0.5 mM ATP and incubated at room temperature for 30 min. cdk2 histone H1 kinase activity was measured as described above in "cdk2 kinase activity assay method".

Purification of CAK

One kg of fresh bovine brain was homogenized in 2 liters of buffer A (20 mM HEPES, 1 mM DTT, 0.3 g/L benzamidine, 0.3 mM PMSF, 0.1 g/L trypsin inhibitor, 2 mg/L pepstatin A, 1 mg/L leupeptin), for 20 sec burst on low speed, 20 sec on medium speed and then 20 sec on high speed at 4°C. The crude homogenate was centrifuged at 12,000 x g for 25 min at 4°C in a Beckman JA-10 rotor and the supernatant was further centrifuged at 120,000 x g for 45 min a 4°C in a Beckman Ti 45 rotor. The 120,000 x g supernatant (1800 ml) was applied to a 350 ml DEAE-Sepharose CL-6B column (4.5 x 15 cm) which was pre-equilibrated in buffer A. After sample application, the DEAE-Sepharose column was washed with 5 bed volumes of buffer A and then eluted with a linear gradient of 0- 0.45 M NaCl in 2 L buffer A at a flow rate of 300 ml/hr. Fractions with activity were pooled (360 ml, total).

The pooled fraction with activity from DEAE-Sepharose was subsequently applied onto a 50 ml Hydroxylapatite column (2.5 x 6 cm) which was pre-equilibrated in buffer A. Followed by washing with 5 bed volumes of buffer A, the Hydroxylapatite column was eluted with a linear gradient of 0-0.4 M K_2HPO_4/KH_2PO_4 in 500 ml buffer A at a flow rate of 80 ml/hr. The activity peak (80 ml) was pooled.

The pooled fraction with activity from the Hydroxylapatite column was further applied onto a 40 ml Heparin Sepharose column (2.5 x 6 cm) which was pre-equilibrated in buffer A. After washing with 5 bed volumes of buffer A, the Heparin Sepharose column was eluted with a linear gradient of 0-0.7 M NaCl in 200 ml buffer A at a flow rate of 60 ml/hr. The activity peak (45 ml) was pooled and dialyzed against buffer A and further concentrated to 10 ml by dialysis against Aquacide II.

The concentrated activity peak sample was finally applied to an FPLC MONO Q column which was pre-equilibrated in buffer A + 50 mM NaCl. The column was washed with 30 ml buffer A + 50 mM NaCl and then eluted with a linear gradient of 50 mM-500 mM NaCl in 30 ml buffer A at a flow rate of 0.5 ml/min.

Phosphorylation of cdk2 by CAK

50 µl of the peak fraction of CAK from the MONO Q column was incubated with 0.13 µg GST-cdk2, 0.21 µg of PA-cyclin A, 20 mM $MgCl_2$ and 0.5 mM [γ-^{32}P]-ATP (500-1000 dpm/pmol) for 2 hr at room temperature. GST-cdk2 was recovered with 20 µl of GSH-Sepharose with end-over-end rotation for 40 min at 4°C. The GSH-Sepharose was then washed with PBS, PBS + 0.35 M NaCl and PBS,

containing 1 mM DTT, 0.25 mM PMSF, 1μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine and 10 μg/ml trypsin inhibitor. After boiling the GSH-Sepharose in SDS buffer the samples were applied on SDS-PAGE, transblotted by Western blot and then subjected to autoradiography.

Purification of Fully Active GST-cdk2/PA-cyclin A

140 μg of GST-cdk2 and 35 μg of PA-cyclin A were incubated with partially purified bovine brain CAK at room temperature for 1 hour in the presence of Mg^{2+}-ATP. After sample loading, 0.1 ml Ni-NTA column was washed with 20 bed volumes of 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole, followed by 20 bed volumes of 20 mM Tris-HCl pH 8.0, 1.0 M NaCl, 25 mM imidazole, 0.5% Tween-20, 0.5% Triton X-100, and finally 20 bed volumes of 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 50 mM imidazole. Histidine-tagged proteins were eluted with 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 150 mM imidazole. The cdk2 histone H1 kinase activity assay and protein concentration determination were performed as described above. The protein kinase activity peak was pooled and dialyzed against 20 mM Tris-HCl pH 8.0, 20 mM NaCl, 0.5 mM EDTA, containing 1 mM DTT, 0.25 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine and 10 μg/ml trypsin inhibitor, using a centracon spin column. Purified enzyme was finally aliquot and then stored with 1 mg/ml BSA at -80°C for further studies.

RESULTS

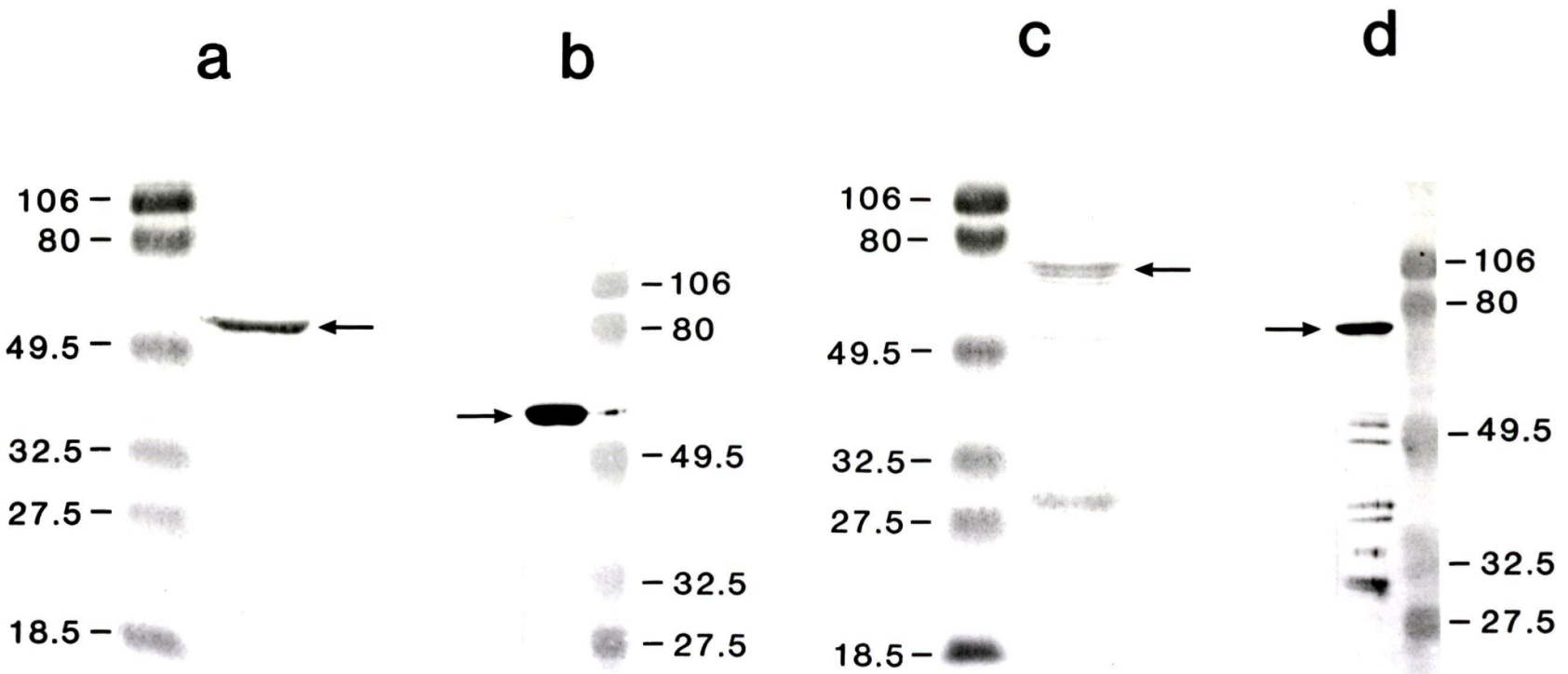
1. Purification of Bacterially Expressed GST-cdk2 and PA-Cyclin A

GST-cdk2 and PA-cyclin A plasmids were obtained from Tim Hunt's lab. Following the plasmid transformation, BL-21 cells were induced by 0.5 mM IPTG at room temperature over night for protein expression. After harvested by centrifugation, cells were lysed by French Press. The 8,000 rpm supernatants of GST-cdk2 or PA-cyclin A were applied on either GSH-Agarose or Ni-NTA Agarose respectively. Elution of GST-cdk2 from GSH-Agarose by 20 mM ß-glutathione, and elution of PA-cyclin A from Ni-NTA Agarose by 150 mM imidazole were described in "Material and Method". Purified GST-cdk2 and PA-cyclin A were analyzed by SDS-PAGE and Western blot.

Highly purified bacterially expressed GST-cdk2 protein is about 60 kDa on SDS-PAGE which was stained with Coomassie Blue (FIG.1, a). Anti-cdk2 C-terminal antibody is directed against the last 12 amino acid residues of $p33^{cdk2}$, a sequence region that is unique in comparison to other cdks. The bacterially expressed GST-cdk2 protein displayed very strong C-terminal immunoreactivity on Western blot (FIG.1, b). The major band of PA-cyclin A protein was found around 80 kDa on Coomassie Blue stained SDS-PAGE (FIG.1, c).

Figure 1. Purification of bacterially expressed GST-cdk2 and PA-cyclin A

Bacterially expressed GST-cdk2 from GSH-Agarose and PA-cyclin A from Ni-NTA-Agarose were analyzed on SDS-PAGE and Western blot. Purified GST-cdk2 (**a**) and PA-cyclin A (**c**) were subjected to SDS-PAGE and then stained with Coomassie blue. Purified GST-cdk2 and PA-cyclin A were applied to SDS-PAGE and subsequently transblotted onto Immobilon-P membranes. After incubation with cdk2 C-terminal antibody (**b**) and human cyclin A antibody (**d**), immunoreactive bands were visualized using an alkaline phosphatase conjugated secondary antibody and NBT/BCIP as substrates.


a
b
c
d
106
80
49.5
32.5
27.5
18.5
106
80
49.5
32.5
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106
80
49.5
32.5
27.5
18.5
106
80
49.5
32.5
27.5

Since PA-cyclin A was a protein A tagged protein, the molecular weight of PA-cyclin A was greater than normal cyclin A protein which is 51 kDa. Antibody towards the human cyclin A protein reacted very well with bacterially expressed PA-cyclin A on Western blot (FIG.1, d). However, several weakly-stained bands between 80 kDa and 27 kDa were found on both SDS-PAGE and Western blot which were thought as the degradative products of PA-cyclin A protein. The immunoreactive properties of GST-cdk2 and PA-cyclin A verified bacterially expressed GST-cdk2 and PA-cyclin A proteins.

2. Purification of CAK From Bovine Brain

As Poon described previously (Poon et al., 1993), reconstitution of purified bacterially expressed GST-cdk2 and PA-cyclin A in the presence of Mg^{2+}-ATP did not lead to the appearance of histone H1 kinase activity. However after preincubation of a HeLa cell extracts with Mg^{2+}-ATP, cdk2 histone H1 kinase activity was obtained (data not shown). This result suggested that a cdk2 activator exists in cell extract which was termed as cdk-activating kinase. *In vitro* studies attempting to reconstitute cdk2 protein kinase activity showed that binding to cyclin A and phosphorylation of threonine 160 were both required to active fully the histone H1 kinase activity of cdk2 (Poon et al., 1993; Fisher et al., 1994; Tassan et al., 1994; Mäkelä et al., 1994). CAK responsible for phosphorylation of cdk2 on Thr 160 was

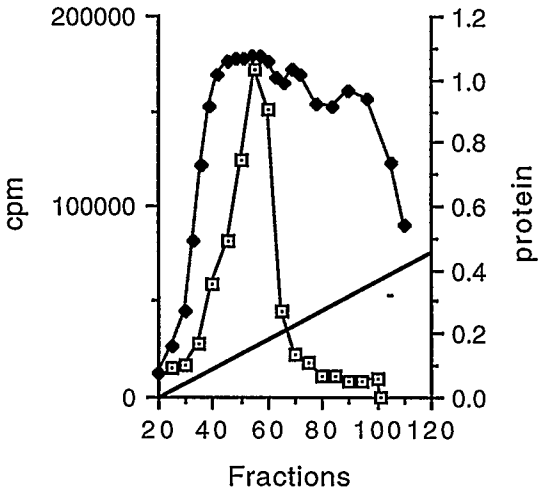
identified and its major catalytic subunit, $p40^{MO15}$, was cloned (Solomon et al., 1992; Shuttleworth et al., 1990). Recently, purification of CAK from Xenopus egg, Starfish oocytes and HeLa cells was reported (Solomon et al., 1993; Fesquet et al., 1993; Fisher et al., 1994; Tassan et al., 1994). Inspired by the above studies and in order to reconstitute the active form of bacterially expressed GST-cdk2/PA-cyclin A kinase, the purification of CAK from bovine brain was carried out, using GST-cdk2/PA-cyclin A as the substrate.

In order to purify CAK from bovine brain, a two-stage assay was employed. CAK activity was followed by its ability to activate GST-cdk2/PA-cyclin A complex: fractions were first incubated with unphosphorylated GST-cdk2/PA-cyclin A complexes and the resulting activated GST-cdk2 kinase was detected by its ability to phosphorylate the substrate histone H1 peptide.

Whole bovine brain was homogenized and the cytosolic fraction was isolated by centrifugation at 120,000x g, as described in "Materials and Methods". CAK activity was barely detectable in this extract. However, after the crude extract was applied to a DEAE-Sepharose column, significant activity of CAK was observed in the NaCl gradient eluted fractions (FIG. 2, a). Peak fractions were pooled, applied onto a Hydroxylapatite column and then eluted with 0-0.4 M K_2HPO_4/KH_2PO_4 gradient (FIG. 2, b). Further purification was obtained by a Heparin-Sepharose column. The pooled fractions of the peak of

Figure 2. Purification of CAK from bovine brain

Fractions from the column chromatography steps in the purification of bovine brain CAK were assayed for activating kinase activity toward GST-cdk2 in the presence of cyclin A. cdk2 histone H1 kinase activity assay and protein concentration determination were performed as described in "Material and Method". The column steps are DEAE-Sepharose (**a**), hydroxylapatite (**b**), Heparin-Sepharose (**c**) and MONO Q (**d**).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100000
0
cpm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
protein
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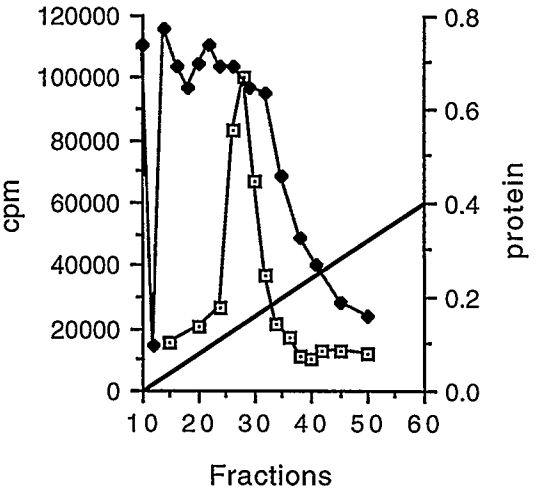
Fractions

a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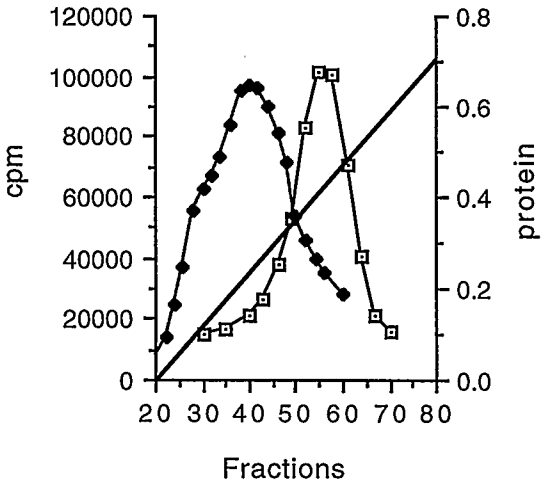
Fractions

b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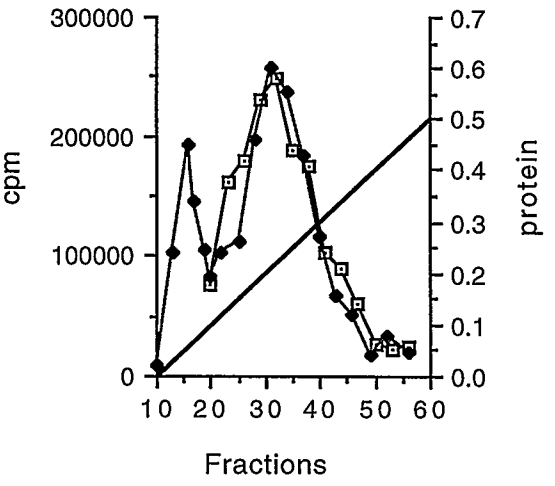
20 30 40 50 60 70 80
Fractions

c


300000
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cpm
0.7
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0.5
0.4
0.3
0.2
0.1
0.0
protein
10 20 30 40 50 60
Fractions

d

activity from Hydroxylapatite column were loaded onto a Heparin-Sepharose column. The bound activity was eluted with 0.7 M NaCl (FIG. 2, c). The pooled activity peak was dialyzed against HEPES buffer and then concentrated. The concentrated peak of activity was finally fractionated on MONO Q column with 0.5 M NaCl gradient (FIG. 2, d).

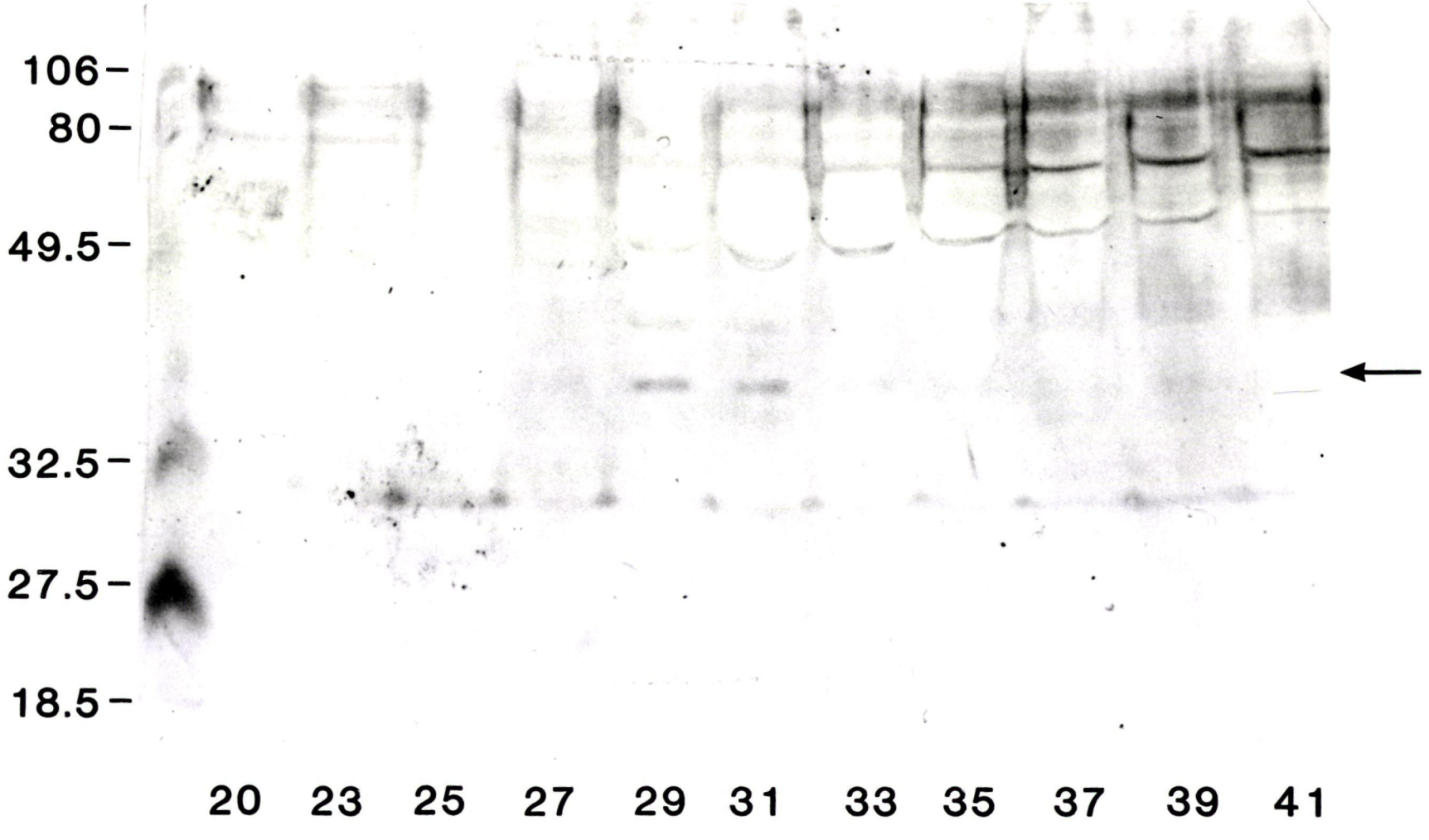
Due to the relative difficulty of the two-step assay, it is difficult to evaluate precisely the extent of CAK purification. However, during the partial purification, it was found that the amount of CAK in brain was rare. This was further suggested by the following observations: CAK activity was not detectable in crude brain extract, whereas a similar amount of HeLa cell extract incubated with GST-cdk2/PA-cyclin A resulted in detectable cdk2 histone H1 kinase activity(data not shown). However, one can not rule out the possibility that CAK inhibitors exist in brain crude extracts and inhibit CAK activity.

3. Immunological Analysis of Bovine Brain CAK

Partially purified CAK fractions from the MONO Q column were analyzed by Western blot using $p40^{MO15}$ polyclone antibody. Xenopus GST-MO15 polyclonal antibody reacted with bovine brain CAK but not very strong (FIG. 3). The profile of immunoreactive staining corresponded exactly to that of CAK kinase activity (FIG. 2, d). This data is indicative of the presence of CAK protein in bovine brain and suggested that bovine brain homologue of $p40^{MO15}$ is the major

Figure 3. Western blot analysis of partially purified CAK from bovine brain with anti-MO15 antibody

Fractions from MONO Q column were subjected to SDS-PAGE and then electroeluted onto Immobilon-P membrane. After incubation with GST-MO15 polyclonal antibody, immunoreactive bands were visualized using an alkaline phosphatase conjugated secondary antibody and NBT/BCIP as substrates.


106-
80-
49.5-
32.5-
27.5-
18.5-
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41

catalytic subunit of CAK. However, the weak immunoreactivity of bovine brain CAK with Xenopus GST-MO15 antibody revealed that there may be some sequence difference between Xenopus $p40^{MO15}$ and bovine brain MO15. In addition, when purified from bovine brain, CAK did not bind to some columns to which Starfish CAK does, such as MONO S, suggesting that bovine brain CAK also differs from Starfish CAK. Because of its function as a cdk-activating kinase, bovine brain CAK may be an isoform compared with other CAK's structures.

4. Activation of GST-cdk2/PA-cyclin A by Bovine Brain CAK

a. Activation of GST-cdk2/PA-cyclin A by Bovine Brain CAK Requires ATP, Mg^{2+}

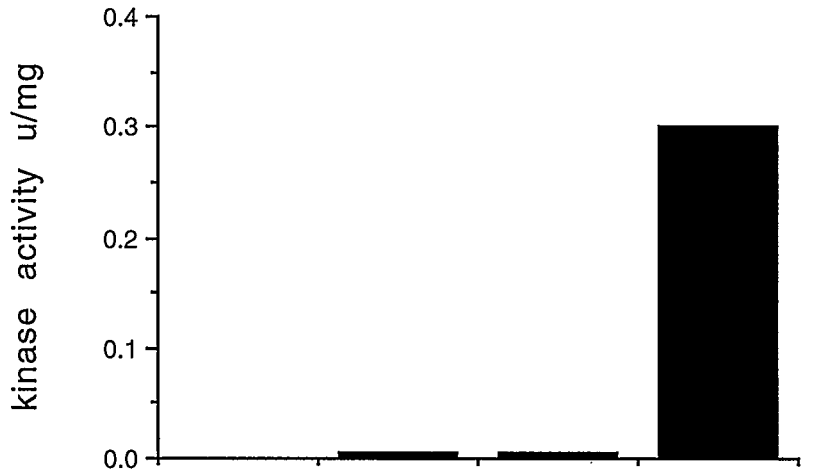
The purification of bovine brain CAK using GST-cdk2/PA-cyclin A as substrate showed that CAK has the ability to activate the GST-cdk2/PA-cyclin complex. Although Mg^{2+}-ATP was added in the preincubation to activate GST-cdk2/PA-cyclin A with CAK, it was not determined whether Mg^{2+}-ATP was required for this activation. The activation of GST-cdk2/PA-cyclin A by bovine brain CAK in the presence or absence of Mg^{2+}-ATP was then investigated. When incubated with brain CAK but without Mg^{2+}-ATP, cdk2 histone H1 kinase activity did not turn on (FIG. 4, lane 3). Only in the presence of Mg^{2+}-ATP during the incubation of GST-cdk2/PA-cyclin A with brain CAK, was cdk2 kinase activity dramatically increased (FIG. 4, lane 4).

This result suggested that bovine brain CAK is a protein kinase rather than a binding activator, and that bovine brain CAK activates GST-cdk2/PA-cyclin A probably by phosphorylation of a certain amino acid residue on cdk2. Besides, these results appeared to be consistent with those obtained in some other experiments before. CAK in Xenopus egg, Starfish oocytes and human HeLa cells were reported as protein kinases and the activation of both $p34^{cdc2}$ and $p33^{cdk2}$ by CAK was Mg^{2+}-ATP dependent(Poon et al., 1993; Solomon et al., 1993; Fesquet et al., 1993; Fisher et al., 1994).

As expected, bacterially expressed GST-cdk2 protein kinase activity was not detectable even in the presence of CAK (FIG. 4, lane 1). GST-cdk2 could bind to PA-cyclin A, however, as previously found by Connell-Crowley et al. (1993) and Poon et al. (1993), even without other additions, the kinase activity of this complex was always slight higher than that of GST-cdk2 alone (FIG. 4, lane 2). Without PA-cyclin A binding, GST-cdk2 failed to achieve histone H1 kinase activity although it was preincubated with CAK (FIG. 4, lane 1). Only the complex of GST-cdk2/PA-cyclin A which was activated by brain CAK showed very strong ability of phosphorylation of the histone H1 peptide (FIG. 4, lane 4). Thus PA-cyclin A was detected as a companion subunit for cdk2 kinase subunits and association of cdk2 with cyclin A appeared to be essential for its protein kinase activity. These results showed the same manner as in the activation of cdk2 by Xenopus CAK (Poon et al., 1993; Fesquet et al., 1993).

Figure 4. Activation of GST-cdk2/PA-cyclin A by bovine brain CAK

Bacterially expressed GST-cdk2 and PA-cyclin A fusion proteins were preincubated alone or together with or without bovine brain CAK in the presence or absence of Mg^{2+}-ATP. Protein kinase activity was assayed as described in "Material and Method".



ATP,Mg^2	+	+	-	+
CAK	+	-	+	+
cyclin A	-	+	+	+
cdk2	+	+	+	+

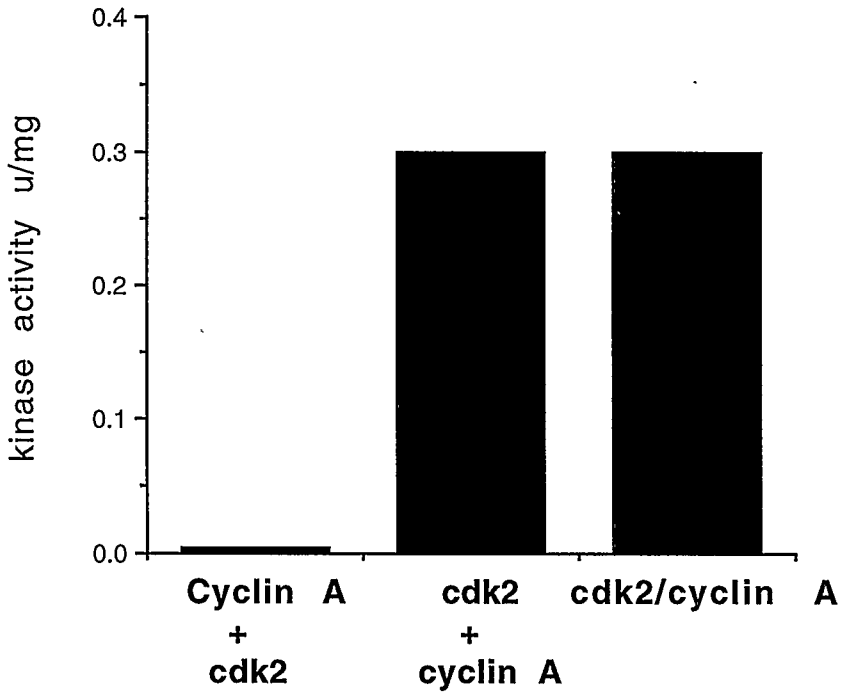
b. CAK Activates GST-cdk2/PA-cyclin A by Phosphorylation of cdk2

To determine if GST-cdk2 or PA-cyclin A is the substrate of bovine brain CAK, the following experiment was designed. GST-cdk2 or PA-cyclin A was incubated with bovine brain CAK separately in the presence of Mg^{2+}-ATP. GST-cdk2 was then harvested on GSH-beads, meanwhile PA-cyclin A was harvested on Ni-NTA-bead. PA-cyclin A was added into cdk2-GSH-bead and GST-cdk2 was added into PA-cyclin A-Ni-NTA-bead right before starting the histone H1 kinase activity assay. The results showed that incubation of GST-cdk2 with CAK first and resulted in histone H1 kinase with PA-cyclin A (FIG. 5, lane 2). In contrast, incubation of PA-cyclin A with CAK followed by histone H1 kinase assay with GST-cdk2 later did not result in the appearance of the kinase activity (FIG. 5, lane 1). These results demonstrated that bovine brain CAK only phosphorylates GST-cdk2. This is also evidence for cdk2 as a protein kinase and cyclin A as a cdk2 activating binding subunit. Phosphorylation of cdk2 but not cyclin A by bovine brain CAK appears to be consistent with the results of phosphorylation of cdk2 by Xenopus CAK (Poon et al., 1993; Fisher et al., 1994; Fesquet, et al., 1993).

c. Phosphorylation of cdk2 by Bovine Brain CAK is Cyclin Independent

Figure 5. Activation of GST-cdk2/PA-cyclin A by phosphorylation of GST-cdk2

Bacterially expressed GST-cdk2 and PA-cyclin A were incubated with bovine brain CAK separately in the presence of Mg^{2+}-ATP. GST-cdk2 was harvested on GSH-bead, meanwhile PA-cyclin A was harvested on Ni-NTA-bead. PA-cyclin A was added to cdk2-GSH-beads and GST-cdk2 was added into PA-cyclin A-Ni-NTA-bead just before starting the kinase activity assay. cdk2 histone H1 kinase activity was assayed as described in "Material and Method".


kinase activity u/mg
0.4
0.3
0.2
0.1
0.0
Cyclin A + cdk2
cdk2 + cyclin A
cdk2/cyclin A

As a protein kinase, bovine brain CAK was showed to be able to activate GST-cdk2/PA-cyclin A kinase by phosphorylation of cdk2. Recently, it has been reported that monomeric cdc2 could not be phosphorylated by CAK. Phosphorylation of cdc2 required cyclin binding to expose the phosphorylatable residue, Thr 161, through the conformational change. Unlike cdc2, monomeric cdk2 could be phosphorylated by CAK without cyclin protein binding (Poon et al., 1993; Fisher et al., 1994). The following question was asked does bovine brain CAK phosphorylate cdk2 in a cyclin-independent manner, as suggested from the results shown in FIG. 5.

After incubation with bovine brain CAK and radioactively labeled [γ-^{32}P]-ATP, GST-cdk2 was harvested on GSH-bead and then analyzed by SDS-PAGE, Western blot with anti-GST antibody and autoradiography. After autoradiography, cdk2 was clearly found to be phosphorylated by brain CAK (FIG. 6a, lane 5). The phosphorylation of GST-cdk2/PA-cyclin A corresponded to the activation of GST-cdk2/PA-cyclin A complex by brain CAK, with the resultsas shown in Figure 4. In contrast, although recombinant bacterially expressed GST-cdk2 and PA-cyclin A could associate to form a stable complex with very low activity, cdk2 was not found to undergo autophosphorylation (FIG. 6a, lane 2).

Monomeric cdk2 catalytic subunit as substrate for bovine brain CAK phosphorylation was also investigated. The results showed that CAK readily phosphorylated monomeric cdk2 (FIG. 6a, lane 4). As expected, purified bacterially expressed GST-cdk2 itself did not show autophosphorylation. (FIG. 6a, lane 1). The phosphorylation of monomeric cdk2 with Xenopus CAK and HeLa cell CAK has also been seen in other experiments (Poon et al., 1993; Fisher et al., 1994). Unlike cdc2, cdk2 phosphorylation appeared not to depend on the presence of cyclin. This can be explained by cdk2 crystal structure analysis. Thr 160 region of the protein is flexible enough, even in the absence of cyclin, to permit CAK access to its substrate (Fisher et al., 1994).

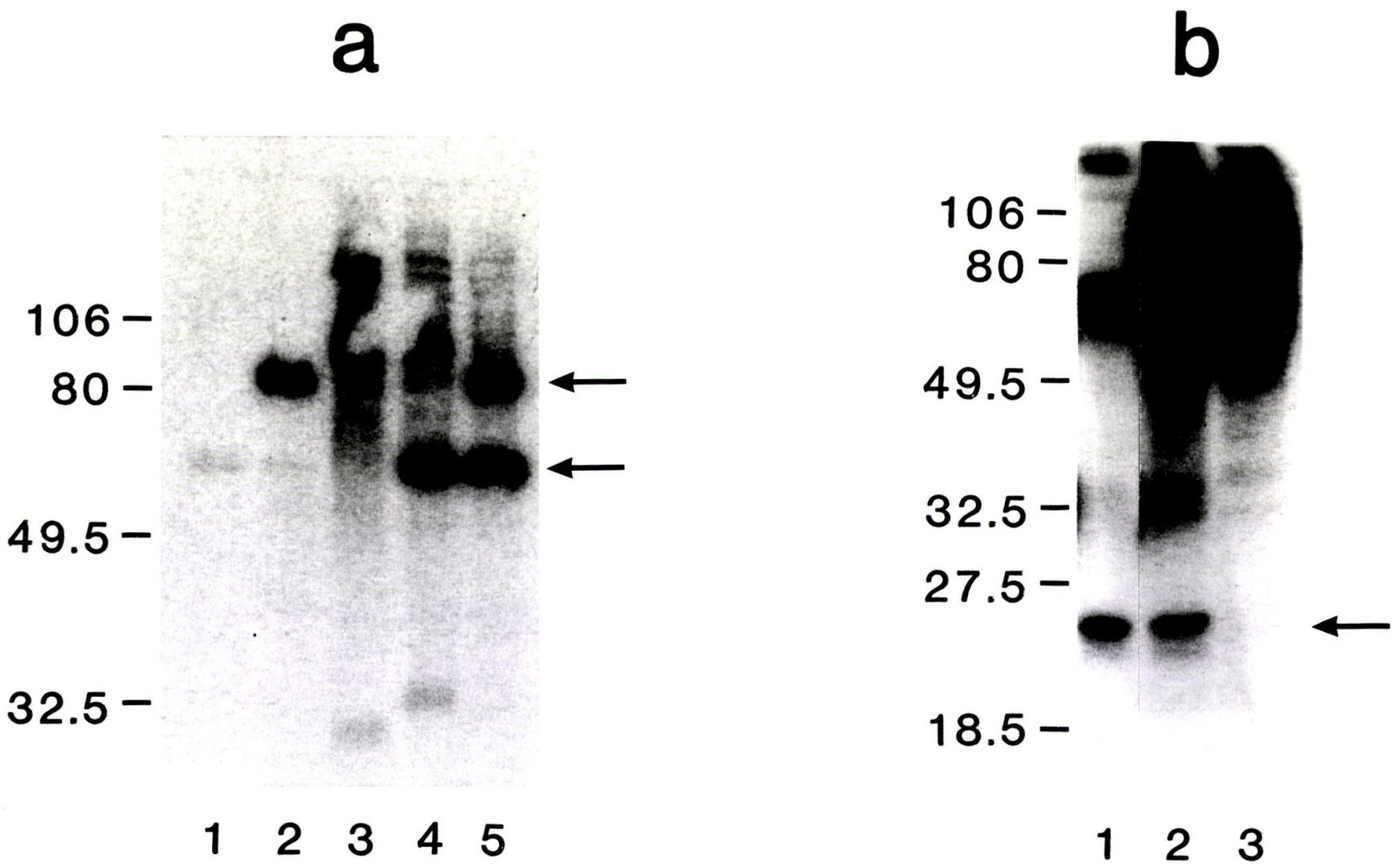
As the substrate of cdk2, PA-cyclin A was found to be phosphorylated by cdk2 with or without bovine brain CAK (FIG. 6a, lane 2 and 5). This suggested that cyclin A was a very good substrate for cdk2 phosphorylation even if when CAK when cdk2 kinase activity was only weakly detected. GST as a control of GST-cdk2 was not shown to be appear either autophosphorylated or phosphorylated by bovine brain CAK at 28 kDa (FIG. 6a, lane 3).

d. Bovine Brain CAK Phosphorylates cdk2 Only on Threonine

$p33^{cdk2}$ is normally phosphorylated on threonine 160 *in vivo* (Gu et al., 1992). *In vitro*, phosphorylations of cdk2 by CAK from Xenopus egg,

Figure 6. Phosphorylation of GST-cdk2 and cdk5 by bovine brain CAK

(**a**) Bacterially expressed GST-cdk2 and PA-cyclin A were incubated alone or together with or without bovine brain CAK with [r-^{32}P]-ATP at room temperature for 1 hour. Reactions were stopped with SDS sample buffer, and proteins were separated by SDS-PAGE and then transblotted onto Immobilon-P membrane. After incubation with GST antibody, immunoreactive bands were visualized using an alkaline phosphatase conjugated secondary antibody and NBT/BCIP as substrates. Phosphorylated proteins were visualized by autoradiography. (**b**) MONO S purified cdk5/p25 was incubated with or without bovine brain CAK with [r-^{32}P]-ATP at room temperature for 1 hour. Reactions were stopped with SDS sample buffer, and proteins were separated by SDS-PAGE and stained with silver. The gel was dried, and phosphorylated proteins were visualized by autoradiography.


a
106
80
49.5
32.5
1 2 3 4 5
b
106
80
49.5
32.5
27.5
18.5
1 2 3

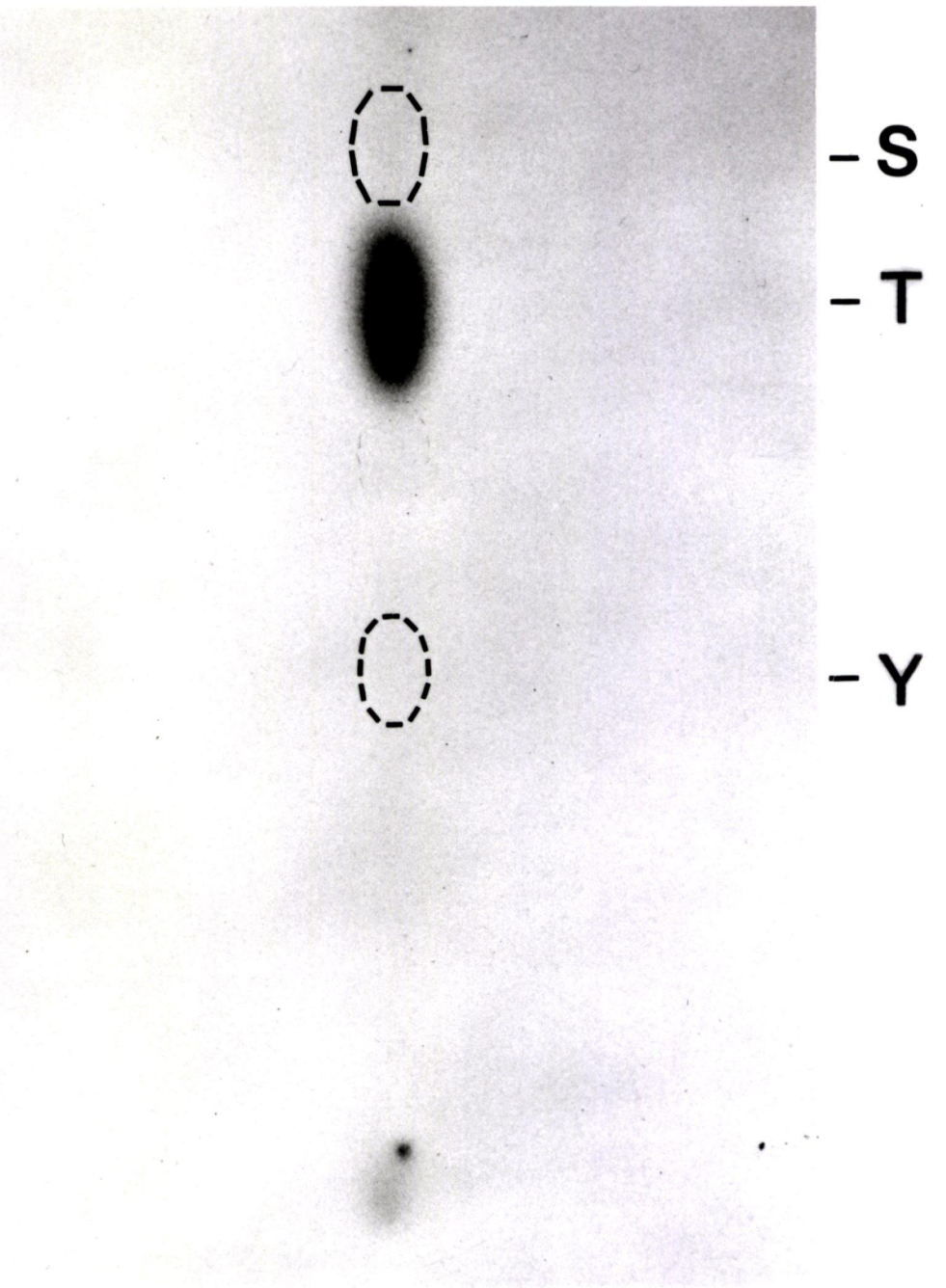
HeLa cells and Starfish occytes were localized on a specific threonine, Thr 160, which is the equivalent site of Thr 161 in $p34^{cdc2}$. Both Thr 161 in $p34^{cdc2}$ and Thr 160 in $p33^{cdk2}$ phosphorylation were essential for their protein kinase activity (Poon et al., 1993; Fesquest et al., 1993; Tassan et al., 1994). To investigate whether bovine brain CAK was acting as a specific threonine kinase of cdk2, one dimensional phosphoamino acid analysis of the radioactively labeled GST-cdk2 was investigated. Figure 7 shows the phosphorylation of GST-cdk2 by bovine brain CAK occurs exclusively on threonine. Bovine brain CAK appears to be able to act as a very specific protein kinase that phosphorylated cdk2 on threonine. It has been reported that the GST-cdk2 construct contains 22 other threonine and 24 serine residues, but only Thr 160 was phosphorylated by Xenopus CAK. Phosphorylation of Thr 160 in cdk2 is absolutely required for its protein kinase activity (Poon et al., 1993). Since bovine brain CAK activates GST-cdk2/PA-cyclin A by phosphorylation of GST-cdk2 on threonine, this phosphorylation may also occur on Thr 160.

5. Purification of Active GST-cdk2/PA-cyclin A Kinase

In order to use the right ratio of GST-cdk2 and PA-cyclin A and the correct amount of bovine brain CAK to obtain fully activated GST-cdk2/PA-cyclin A kinase, GST-cdk2, PA-cyclin A and bovine brain CAK activation dose dependent experiments were done. Bacterially expressed GST-cdk2/PA-cyclin A was found to be maximally

Figure 7. Phosphoamino acid analysis of GST-cdk2

One dimensional phosphoamino acid analysis of the radioactively labeled GST-cdk2 band form Figure 6, lane 5 was investigated. The positions of internal marker phosphoserine (**S**), phosphothreonine (**T**) and phosphotyrosine (**Y**) are indicated.


S
T
Y

activated to 0.3 μmol/min/mg. Since purified cdk2/cyclin A is expected to have a specific kinase activity of a few μmol/min/mg, the result suggests that less than 10% of GST-cdk2 could be fully activated. Since PA-cyclin A was found to bind to GST-cdk2 completely, after activated by bovine brain CAK, the GST-cdk2/PA-cyclin A complex was purified from a histidine affinity column, Ni-NTA. 140 μg of GST-cdk2 and 35 μg of PA/cyclin A were incubated with bovine brain CAK in the presence of Mg^{2+}-ATP at room temperature for 1 hour. The phsophorylated GST-cdk2/PA-cyclin A complex was then bound to Ni-NTA Agarose. After washing the column buffer, the protein kinase activity was eluted with 150 mM of imidazole. GST-cdk2/PA-cyclin A was eluted out quickly form a 0.1 ml of Ni-NTA column (FIG. 8). After pooled the activity peak, the purified enzyme was dialyzed against HEPES buffer using contracon spin column. The protein concentration was then estimated by both absorbance at 280 nm and absorbance at 595 nm with Coomassie blue. The aliquots were stored at -80°C with 1 mg/ml BSA for the further studies. The final fully activated GST-cdk2/PA-cyclin A protein kinase had a specific activity of 1.9 μmol/min/mg. This represents a 6-fold purification with a recovery of 40% (Table 1).

6. SDS-PAGE and Immunological Analysis of Fully Active GST-cdk2/PA-cyclin A

Figure 8. Ni-NTA Agarose chromatography

Bacterially expressed GST-cdk2 and PA-cyclin A were incubated with bovine brain CAK with the presence of Mg^{2+}-ATP. Fully activated GST-cdk2/PA-cyclin A kinase was further purified from Ni-NTA Agarose. cdk2 histone H1 kinase activity assay and protein concentration determination were performed as described in "material and method".

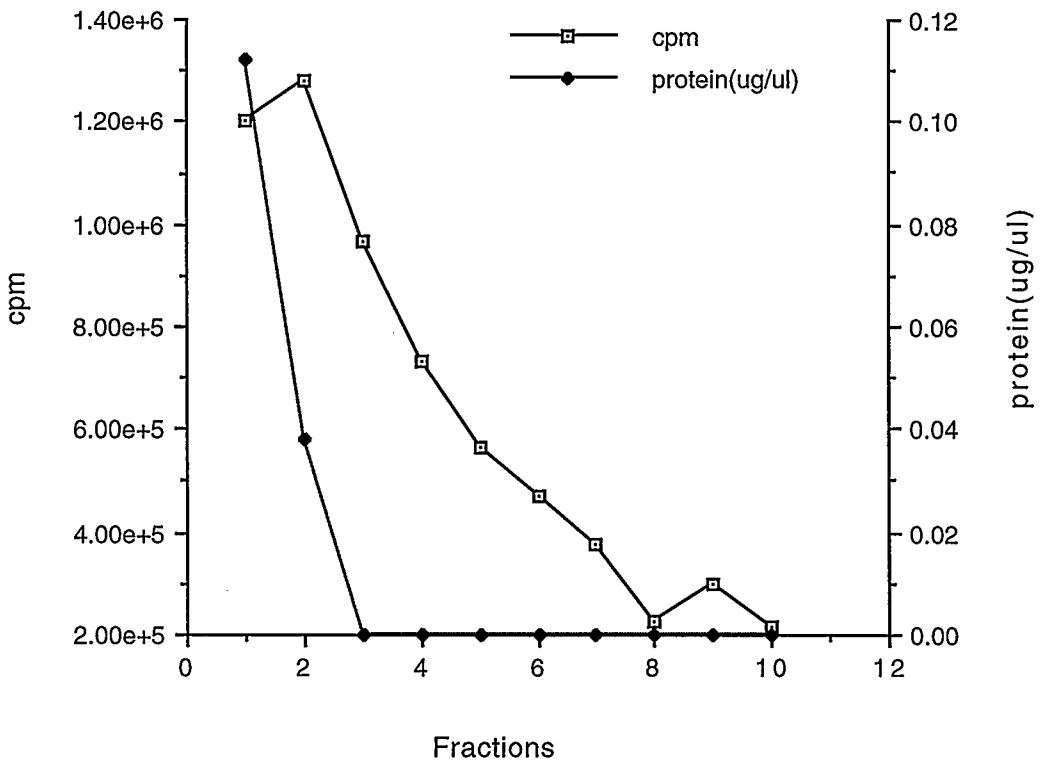

cpm
protein(ug/ul)
1.40e+6
1.20e+6
1.00e+6
8.00e+5
6.00e+5
4.00e+5
2.00e+5
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
12
Fractions

Table 1. Purification of Active cdk2/cyclin A Kinase

	Protein (*μg*)	**Total Activity** (*μmol/min.*)	**Specific Activity** (*μmol/min./mg*)	**Purification** (*fold*)	**Yield** (*%*)
Reconstituted cdk2/cyclin A	175	53	0.3		
Ni-NTA	10.9	21	1.9	6	40%

Figure 9. SDS-PAGE analysis of purified fully active GST-cdk2/PA-cyclin A

GST-cdk2/PA-cyclin A kinase activity fractions from Ni-NTA Agarose were subjected to SDS-PAGE and stained with silver (**a**). After pooling the kinase activity peak, the purified enzyme was dialyzed and aliquoted for further studies. An aliquot of GST-cdk2/PA-cyclin A kinase was applied to SDS-PAGE and stained with silver (**b**).

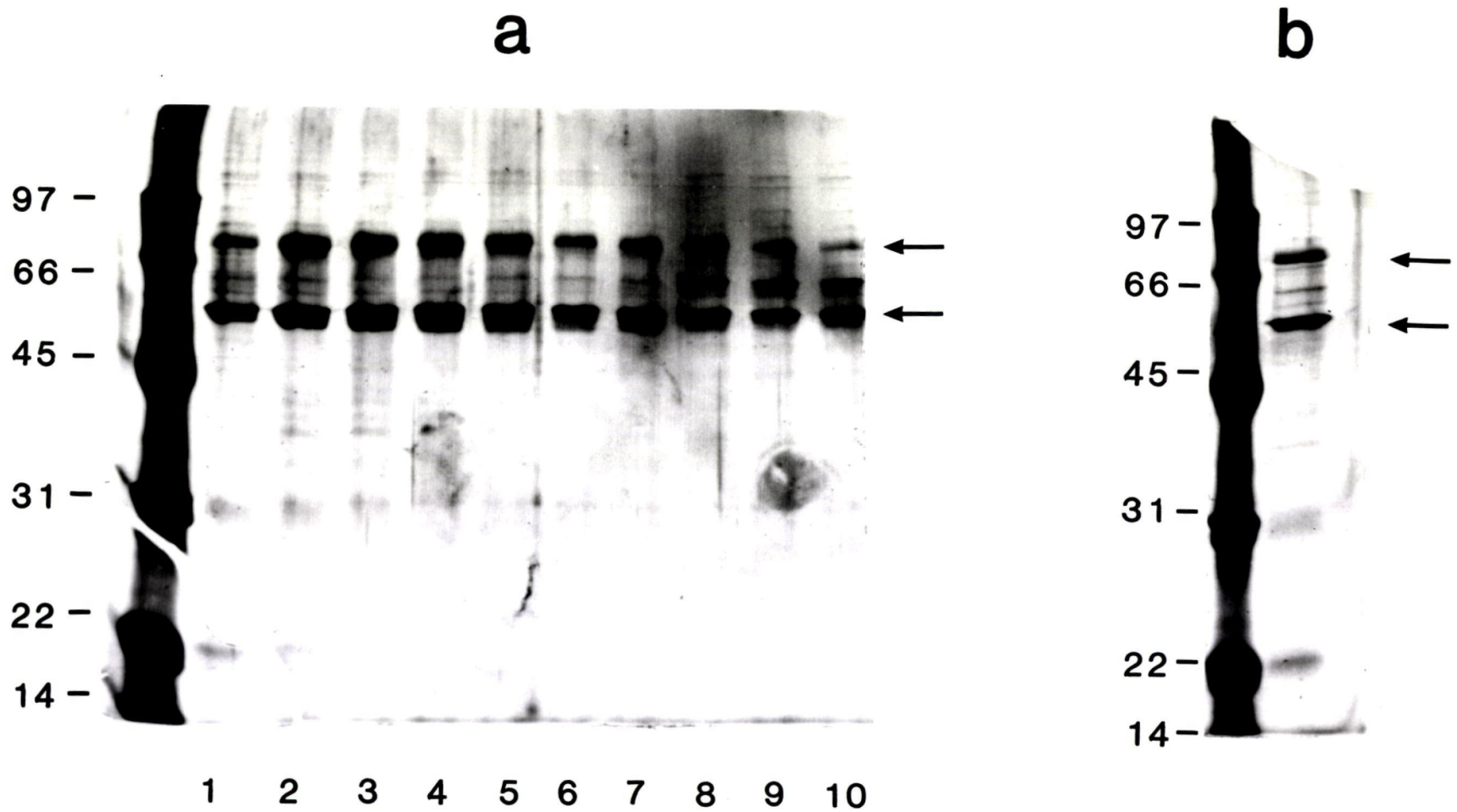
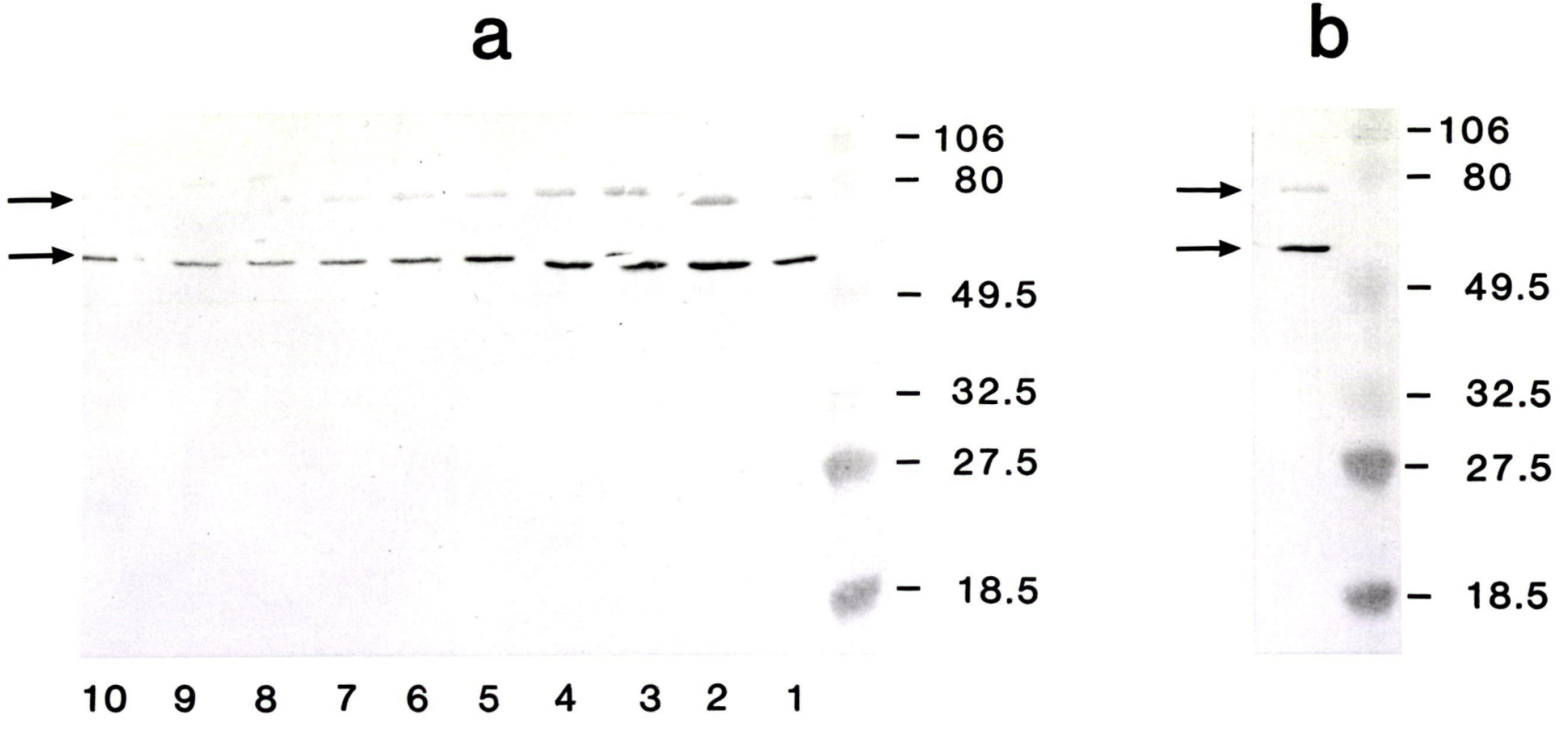

a
b
97
66
45
31
22
14
1
2
3
4
5
6
7
8
9
10

Figure 10. Western blot analysis of purified fully active GST-cdk2/PA-cyclin A kinase with anti-cdk2 C-terminal antibody

Purified fully activated GST-cdk2/PA-cyclin A fractions from Ni-NTA Agarose were subjected to SDS-PAGE and subsequently transblotted onto Immobilon-P membrane. After incubation with cdk2 C-terminal antibody, immunoreactive bands were visualized using an alkaline phosphatase conjugated secondary antibody and NBT/BCIP as substrates (**a**). After pooling the kinase activity peak, the purified enzyme was dialyzed and aliquoted for further studies. An aliquot of GST-cdk2/PA-cyclin A kinase was analyzed on Western blot with anti-cdk2 C-terminal antibody (**b**).


a
b
106
80
49.5
32.5
27.5
18.5
10 9 8 7 6 5 4 3 2 1

Fractions from the Ni-NTA column and the dialyzed enzyme were analyzed by SDS-PAGE and Western blot. Silver stained SDS-PAGE shows that purified enzyme from Ni-NTA contains only GST-cdk2 at 60 kDa and PA-cyclin A at 80 kDa (FIG. 9, a and b). GST-cdk2 was eluted from the PA-cyclin A histidine affinity column, Ni-NTA, confirming that GST-cdk2 can bind to PA/cyclin A to form a stable complex. Anti-cdk2 C-terminal antibody showed very strong immunoreactivity with the purified fully activated GST-cdk2 and PA-cyclin A on Western blot (FIG. 10, a and b). The profile of immunoreactive staining corresponded exactly to that of the proteins seen on the silver stained gel (FIG. 9, a), as well as to that of the kinase activity (FIG. 8).

7. Substrate Sequence Specificity

The substrate recognition motif for $p34^{cdc2}$ is thought to comprise the sequence: -X-S/T-P-X-K/R- based on the analysis of *in vitro* and *in vivo* phosphorylation sites of numerous $p34^{cdc2}$ substrates (Maller, 1990; Moreno and Nurse, 1990). Substrate specificity of a cdc2-like protein kinase, nclk, purified from bovine brain was characterized by Beaudette (1993) using synthetic peptides derived from histone H1. The histone H1 peptide was found to be a specific and unique peptide substrate of cdc2-like kinase. Both bovine brain nclk and HeLa cell $p34^{cdc2}$ displayed similar synthetic peptide substrate specificity (Beaudette et al., 1993). However, with the existence of multiple

forms of cdc2 kinases and cyclins, and evidence to suggest that different forms have different *in vitro* substrate specificity, cdk2/cyclin A kinase substrate specificity was investigated using the fully activated recombinant GST-cdk2/PA-cyclin A complex.

HS(9-18), P-K-T-P-K-K-A-K-K-L, was chosen as the parent peptide to investigate the effect of single amino acid changes or deletions either N-terminal to the phosphorylatable residue, within the proposed $p34^{cdc2}$ consensus sequence or C-terminal to the consensus sequence, on the ability of cdk2/cyclin A to phosphorylate these peptide substrates. This peptide, P-K-T-P-K-K-A-K-K-L, was found to be an excellent substrate for cdk2/cyclin A kinase, having a Km value in the micromolar range (Table 2). There are two residues, a proline and a lysine that are N-terminal to the phosphorylatable residue. Substitution of proline for an alanine, HS(9-18)A9, did not result in a increase in Km values, compared with the parent peptide (Table 2). Unlike brain nclk, the proline at the -2 position did not appear to contribute to efficient significant substrate phosphorylation by cdk2/cyclin A. Further deletion of a lysine residue resulted in a peptide, HS(11-18), showing an 106-fold increase in Km value. The effect of the lysine deletion could effectively be reversed if the α-amino group of the threonine residue was acetylated (Table 2). The result suggested that the lysine residue at the -1 position does not contribute directly as a positive determinant, but neutralizes the strong negative influence of the free α-amino group of

the target threonine residue. This suggestion was supported by the observation that substitution of the lysine residue at the -1 position by an alanine, HS(9-18)A10, does not significantly change the substrate activity of the peptide (Table 2).

The synthetic peptide approach is especially useful in testing proposed sequence motif for substrate activity of protein kinases. Threonine 11 is the residue that can be phosphorylated. Substitution of the threonine for an alanine, HS(9-18)A11, caused inactivation of the peptide as a substrate for cdk2/cyclin A (Table 2). An earier study has already demonstrated the dependence on proline residues at the +1 position for the substrate activity of peptide brain nclk (Beaudette et al., 1993). Similarly, when proline 12 of HS(9-18) was substituted with an alanine, the peptide HS(9-18)A12 was essentially inactive as a substrate for cdk2/cyclin A (Table 2).

The importance of a positively charged residue at position +3 displays a similar role for cdk2/cyclin A. Substitution of lysine 14 by alanine, HS(9-18)A14, resulted in more than 74-fold increase in Km value of the peptide (Table 2). It has been proposed that a polar residue at the +2 position of the consensus motif for $p34^{cdc2}$ is favored for efficient substrate phosphorylation (Moreno and Nurse, 1990) and this was verified by testing the brain nclk (Beaudette et al., 1993). When the lysine at +2 position of the parent peptide HS(9-18) was changed to an alanine, the Km value for HS(9-18)A13

Table 2. Kinetic Parameters of Reconstituted Active cdk2/cyclin A Kinase

Peptide	**Sequence**	**cdk2/cyclin A** *Km (μm)*	***Brain PDK** *Km (μm)*
HS (9-18)	P-K-T-P-K-K-A-K-K-L	4.5	6
HS (9-18)A9	A-K-T-P-K-K-A-K-K-L	5.3	24
HS (9-18)A10	P-A-T-P-K-K-A-K-K-L	3.5	4
HS (9-18)A11	P-K-A-P-K-K-A-K-K-L	no activity	no activity
HS (9-18)A12	P-K-T-A-K-K-A-K-K-L	no activity	no activity
HS (10-18)	K-T-P-K-K-A-K-K-L	53	57
HS (11-18)	T-P-K-K-A-K-K-L	476	500
HS (11Ac-18)	Acety-T-P-K-K-A-K-K-L	20	24
HS (9-18)A14	P-K-T-P-K-A-A-K-K-L	333	376
HS (9-18)A13	P-K-T-P-A-K-A-K-K-L	53	50
HS (9-18)D13	P-K-T-P-D-K-A-K-K-L	500	649
HS (9-18)A16	P-K-T-P-K-K-A-A-K-L	7	26
HS (9-18)A17	P-K-T-P-K-K-A-K-A-L	8	27
HS (9-18)A16,A17	P-K-T-P-K-K-A-A-A-L	83	155

---

*Data from Beaudette et al., 1993

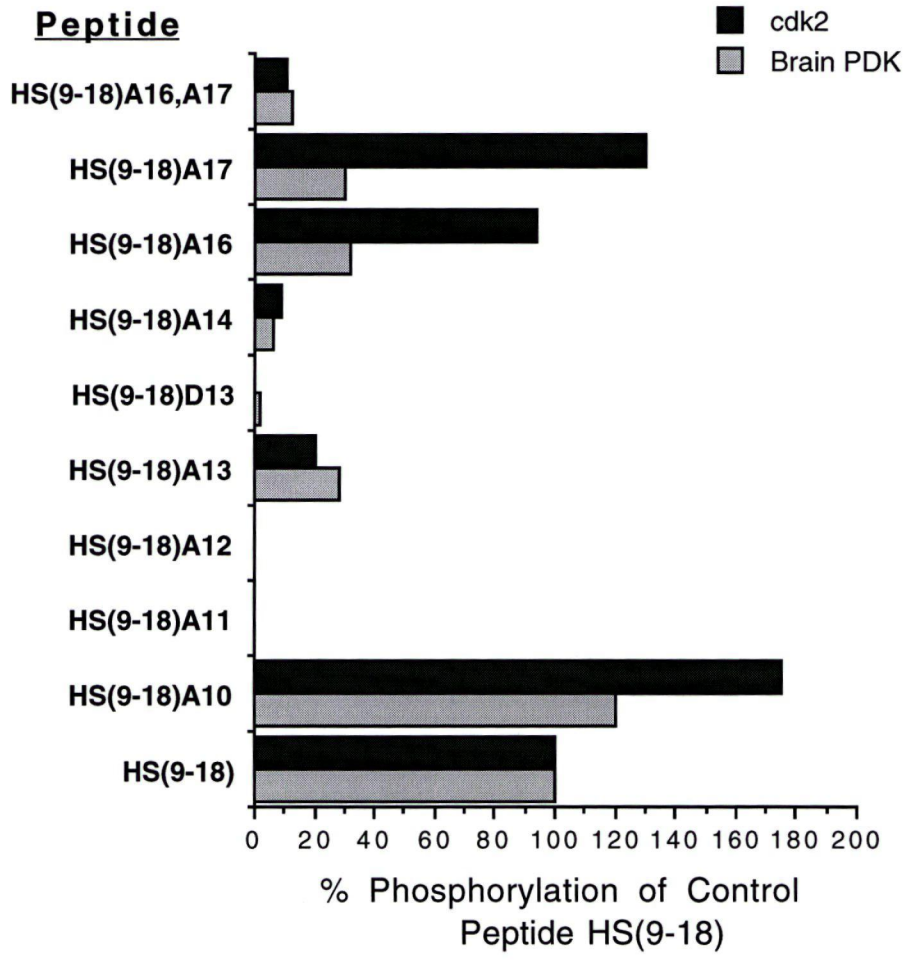
increased 12-fold for cdk2/cyclin A. If the same amino acid was substituted by a negatively charged residue, HS(9-18)D13, the Km value increased 111-fold relative to the parent peptide (Table 2). Clearly, a positively charged amino acid is greatly favored over a negatively charged residues at +2 position for the cdk2/cyclin A.

The sequence of basic residues in the proposed $p34^{cdc2}$ consensus motif is clearly important for efficient substrate phosphorylation by cdk2/cyclin A kinase. To investigate the significance of the C-terminal basic cluster in HS(9-18), lysine residues at +5 and +6 positions relative to the phosphorylatable residue were substituted by alanine. Substitution of either lysine residue, HS(9-18)A16 or HS(9-18)A17, resulted in a 1.6-fold or 1.8-fold increase in Km value. Substitution of both lysine residues, HS(9-18)A16,A17, caused a further increase in Km to a value 18 fold higher than the parent peptide (Table 2). C-terminal lysines are important for brain nclk to bind substrate efficiently. However, these secondary positive substrate determinants do not appear to play significant roles in the substrate activity of the peptide for cdk2/cyclin A.

Furthermore, the initial rates of phosphorylation of the various peptide analogues at a single peptide concentration for cdk2/cyclin A were compared with that of for brain nclk. Figure 11 represents initial rates of phosphorylation for both cdk2/cyclin A and brain nclk with histone peptides having amino acid substitutions within the

Figure 11. Relative rates of phosphorylation by cdk2/cyclin A and bovine brain nclk for histone peptide derivatives having changes in the proposed $p34^{cdc2}$ consensus sequence and having basic amino acid residues substituted

The phosphorylation of synthetic peptides (5 μM) was performed with purified fully activated GST-cdk2/PA-cyclin A or purified bovine brain nclk as described in "material and method". Rates of phosphorylation are expressed as percentage on the parent peptide HS(9-18). Black bars represent phosphorylation reactions performed with cdk2/cyclin A, and hatched bars represent phosphorylation reactions performed with purified bovine brain nclk.


Peptide
cdk2
Brain PDK
HS(9-18)A16,A17
HS(9-18)A17
HS(9-18)A16
HS(9-18)A14
HS(9-18)D13
HS(9-18)A13
HS(9-18)A12
HS(9-18)A11
HS(9-18)A10
HS(9-18)
0 20 40 60 80 100 120 140 160 180 200
% Phosphorylation of Control Peptide HS(9-18)

proposed S/T-P-X-K/R motif. Relative rates of phosphorylation are expressed as a percentage of the parent peptide HS(9-18). As expected, cdk2/cyclin A kinase activity was proline dependent and required the presence of a basic residue in the +3 position of the substrate peptides for favorable phosphorylation. Also, the phosphorylatable residue, threonine, was essential for the peptidephsophorylation activity (FIG. 11). As is the case with the brain nclk, a basic amino acid residue at the +2 position is clearly favored over an acidic or neutral residue, as relative rates of phosphorylation for cdk2/cyclin A with the peptides having an aspartic acid residue or alanine in place of lysine were undetectable and 25% of the parent peptide, respectively (FIG. 11).

In addressing the role of basic residues outside the proposed consensus motif, cdk2/cyclin A and brain nclk appear to have slightly different requirements for the basic cluster downstream from the phosphorylation site. Substitution of a lysine residue in HS(9-18) at the +5 or +6 position did not cause a drop of relative rates of phosphorylation relative to the parent peptide. Substitution of both residues drops the relative rate of phosphorylation to approximately 20% of the parent peptide. However, for brain nclk, lysine residues at +5 and +6 positions are important basic residues downstream from the consensus sequence for efficient substrate binding. The lysine residue immediately N-terminal to the phosphorylation threonine in HS(9-18) did not appear to be important determinant, as substitution

of this residue for alanine resulted in a 80% increase in relative rates of phosphorylation for cdk2/cyclin A. This result revealed the same role as substitution of N-terminal lysine for brain cdc2-like kinase (FIG. 11).

8. Bovine Brain CAK does not Activate or Phosphorylate cdk5/p25

Cyclin-dependent kinase 5, cdk5, was identified both as the catalytic subunit of a novel bovine brain protein kinase or by screening human cDNA library based on its close primary sequence homology to the human cdc2 kinase (Lew et al., 1992; Meyersone et al., 1992). cdk5 was found at the highest level in brain, at intermediate level in testis, and low or undetectable levels in all other tissues, but brain is the only tissue that shows cdk5 histone H1 kinase activity (Tsai et al., 1993). A novel regulatory subunit, p25, was found to associate with cdk5 (Lew et al., 1992). Unlike cdk5, the p25 transcript is expressed only in brain. When overexpressed and purified from *E. Coli*, p25 can activated cdk5 *in vitro*. However, unlike the case with other cyclin-dependent kinases, full activation of cdk5 occurs upon binding p25 alone without the requirement for exogenous phosphorylation (Lew et al., 1994). Because one can not rule out the possibility that phosphorylation at Ser 159 in cdk5, the equivalent site to Thr 161 in $p34^{cdc2}$, may result in further activation of the

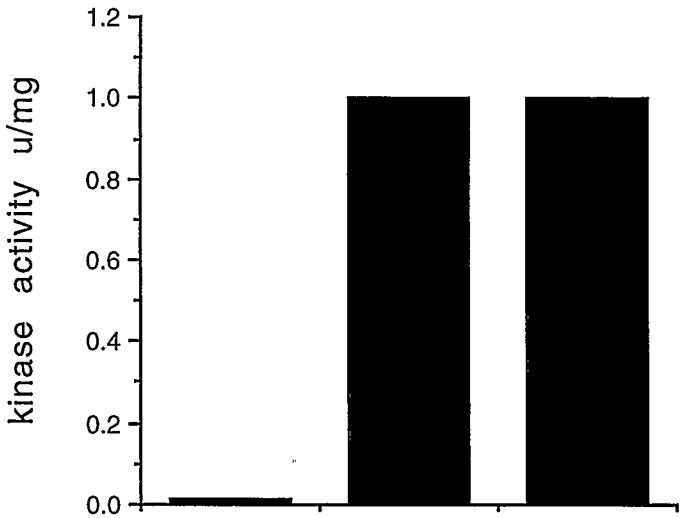
enzyme, the activation and phosphorylation of cdk5/p25 by bovine brain CAK was investigated.

GST-cdk5 and GST-p25 fusion proteins were individually expressed in *E. Coli*, purified by glutathione-agarose chromatography and then reconstituted at room temperature for 2 hours for determination of kinase activity. Highly activated cdk5/p25 was obtained from a MONO S column after thrombin digestion of GST (Data not shown). When incubated with bovine brain CAK in the presence of Mg^{2+}-ATP, cdk5/p25 protein kinase activity was determined using histone H1 peptide. The protein kinase activity of cdk5/p25 did not increase when the brain CAK was present (FIG. 12, lane 3). The histone H1 kinase activity of cdk5/p25 appeared to be similar to that of without bovine brain CAK (FIG. 12, lane 2). In contrast, GST-cdk2/PA-cyclin A kinase activity dramatically increased with incubation of bovine brain CAK (FIG. 4, lane 4). This demonstrated that fully activated cdk5 protein kinase activity only requires binding with its activator, p25, but does not required phosphorylation by brain CAK. This is also supported by the observations that purification of reconstituted cdk5/p25 results in an enzyme specific activity which is similar to that of neuronal cdc2-like kinase purified from brain (Lew et al., 1994).

To further understand the mechanism of activation of cdk5, phosphorylation of cdk5 by bovine brain CAK was subsequently

Figure 12. Effect of bovine brain CAK on histone kinase activity of cdk5/p25

MONO S purified highly activated cdk5/p25 was incubated with or without bovine brain CAK in the presence of Mg^{2+}-ATP. cdk5 histone H1 kinase activity was assayed as described in "material and method".



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* A reconstituted active cdk5/p25 which is purified from MONO S.

investigated. When incubated with radioactively labeled [γ-^{32}P]-ATP, cdk5 was not found to be either autophosphorylated or phosphorylated by brain CAK at 32 kDa (FIG. 6b, lane 2). p25 appeared to be highly phosphorylated by active cdk5 (FIG. 6b, lane 1). However, phosphorylation of p25 did not appear to increase when with the presence of bovine brain CAK (FIG. 6b, lane 2). That brain cdk5 can not be activated and phosphorylated by brain CAK represents a newpattern of activation of cyclin-dependent kinase activity. It also suggested that bovine brain CAK may have some other functional activity in brain instead of phosphorylation of cdk5 and have some other substrates in brain rather than cdk5 kinase.

DISCUSSION

1. Bovine Brain CAK Contains p40^{mo15}-Related Subunit

The p34^{cdc2} protein kinase and structurally related cdks play a pivotal role in promoting cell cycle progression in all eukaryotes. In addition to cell cycle-dependent changes in the subunit composition of cdk/cyclin complexes, the reversible phosphorylation of p34^{cdc2} and the other cdks has been recognized as a major mechanism for regulating their activities. Since the phosphorylation state of cdks appears to be controlled, it is important to identify the kinases and phosphatases acting on cdks, and to determine how their activities are integrated with intracellular signal transduction pathways. Important progress in this field has been made recently with the identification of p40^{MO15} as the catalytic subunit of cdk-activating kinase (CAK), the enzyme responsible for phosphorylating p34^{cdc2} on Thr 161 (Solomon et al., 1993; Poon et al., 1993; Fesquet et al., 1993). This enzyme is also able to phosphorylate a corresponding residue in p33^{cdk2}, Thr 160, and possibly other cdks (Draetta 1993; Solomon 1994).

In the present work, cdk-activating kinase has been partially purified from bovine brain using cdk2/cyclin A as substrate. This is the first evidence of presence of CAK in brain. Purification of bovine brain CAK protocol is different from that of Starfish CAK since bovine brain CAK does not bind to a MONO S column which is one of

the chromatography steps for purification of Starfish CAK (Fisquet et al., 1993). This suggests that bovine brain CAK displays a different structure from Starfish CAK.

The $p40^{MO15}$ protein was initially identified during a search for Xenopus sequences that were related to human $p34^{cdc2}$ (Shuttleworth et al., 1990). Peptide sequencing of proteins from extensively purified CAK from Xenopus or nearly purified CAK from Starfish showed that $p40^{MO15}$ or a very closely related protein was present in the most enriched fractions. Immunoprecipitation demonstrated that $p40^{MO15}$ copurified with CAK activity, and immunoblotting showed that CAK was recognized by an antibody to $p40^{MO15}$ (Solomon et al., 1993; Poon et al., 1993; Fesquet et al., 1993). Studies of the properties of $p40^{MO15}$ fused to glutathione S-transferase (GST-$p40^{MO15}$) showed that 'naked' GST-$p40^{MO15}$ had no CAK activity, but it became active after incubation with a Xenopus egg extract (Poon et al., 1993). Studies of purification of human CAK and expression from a cloned human $p40^{MO15}$ gene also confirmed that human CAK contains a $p40^{MO15}$ closely related protein (Tassan et al., 1994; Fisher et al., 1994). Antibody raised against bacterially expressed Xenopus $p40^{MO15}$ readily immunoreacts with bovine brain CAK indicated that bovine brain CAK contains a protein identical or closely related to $p40^{MO15}$ as its catalytic subunit. However, immunoprecipitation of bovine brain CAK failed to achieve its protein kinase activity using Xenopus $p40^{MO15}$ antibody. Taken

together, these results show that bovine brain CAK contains a functional homologue of $p40^{MO15}$ and the sequence of brain $p40^{MO15}$ may be distinct from Xenopus $p40^{MO15}$ or Starfish $p40^{MO15}$, though brain CAK is suspected as an isoform kinase.

2. Bovine Brain CAK is a Protein Kinase

Since Thr 161 of $p34^{cdc2}$ aligns with an autophosphorylation site found in some protein kinases, it had been suspected that $p34^{cdc2}$-like proteins might also autophosphorylate, making CAK an activator of autophosphorylation, rather than a protein kinase. This appears not to be the case. Activation of cdk2/cyclin A by bovine brain CAK is Mg^{2+}-ATP dependent. When incubated brain CAK was interacted with cdk2/cyclin A in the absence of Mg^{2+}-ATP, cdk2 histone H1 kinase activity was not increased. cdk2/cyclin A kinase activity was obtained only in the presence of Mg^{2+}-ATP when incubated with brain CAK. Moreover, cdk2 was not found to undergo autophosphorylation.

It was reported previously that CAK phosphorylated $p34^{cdc2}$ in a strictly cyclin-dependent manner. In contrast, phosphorylation of $p33^{cdk2}$ by CAK did not require association with cyclins (Poon et al., 1993; Fisher et al., 1993; Solomon et al., 1993). Phosphorylation of cdk2 by brain CAK was found to be cyclin-independent manner. Monomeric cdk2 was readily phosphorylated by brain CAK, however,

binding to cyclin A was essentialfor cdk2 histone H1 kinase activity. The activating Thr 160 residue of cdk2 is largely inaccessible to solvent in the crystal structure of cdk2; however, it is contained within a region of the protein (the T-loop) that is quite flexible. It was proposed that cyclin binding might expose Thr 160 for phosphorylation by CAK through a conformational change in the flexible loop (De Bondt et al., 1993; Morgan and De Bondt, 1994). In the present work, phosphorylation of monomeric cdk2 by brain CAK indicates that the T loop of cdk2 may be flexible enough, even in the absence of cyclin A, to permit CAK access to its substrate.

Bovine brain CAK activated the cdk2/cyclin A complex by phosphorylation of cdk2. This was found by incubation of cdk2 with brain CAK first and then analysis of its histone H1 kinase activity with cyclin A. Subsequently, phosphoamino acid analysis showed that brain CAK phosphorylated cdk2 exclusively on a threonine residues and the phosphorylation of this threonine residue was required for cdk2 protein kinase activity. These results are correlated with the results from analysis of site-directed mutation of Thr 160 in $p33^{cdk2}$; a T160A mutant could not be phosphorylated and activated by CAK (Poon et al., 1993).

Although bovine brain CAK was partially purified and characterized, it is still unknown whether brain CAK contains some other association subunits. Purification of CAK from Xenopus,

Starfish and human HeLa cells showed that there was another major subunit associated with $p40^{MO15}$ and required for CAK activity. The second subunit from human has been found to be related to the cyclins and is termed cyclin H (Fisher et al., 1994). Cyclin H was also identified in a two-yeast hybrid screen for proteins that interact with $p40^{MO15}$ (Mäkelä et al., 1994). Further purification of brain CAK may identify some other subunits which interacts with the brain homologue of $p40^{MO15}$ to regulate brain CAK activity.

3. Bovine Brain CAK May Have Other Substrates Rather Than cdk2

Bovine brain CAK was purified by its ability to phosphorylate and activate cdk2 histone H1 kinase activity *in vitro* . However, *in vivo* there is no cdk2 in brain (Meyerson et al., 1992) and $p34^{cdc2}$ is not apparently expressed in adult brain either (Hayes et al., 1991). Then the following question of what are the *in vivo* brain CAK's substrates raises. It has been shown that brain does contain anti-$p34^{cdc2}$ reactive material of similar molecular weight on Western blot, suggesting that a similar enzyme is present in this tissue (Draetta et al., 1988). Recently a brain cdc2-like kinase was purified in our lab which consists of a 33-kDa catalytic subunit and a 25-kDa subunit. The 33-kDa catalytic subunit is 58% and 59% amino acid identity to the human $p34^{cdc2}$ and $p33^{cdk2}$, respectively (Lew et al., 1992). In addition it shares 99% amino acid identity with a human

cDNA clone referred to as PSSALRE having only two amino acid residues that are substituted from the bovine clone (Meyerson et al., 1992). More recently, a cdc2-like kinase (cdk5) from a rat brain cDNA library was cloned and which 58% identical to mouse cdc2 and localized at high levels in terminally differentiated neurons (Hellmich et al., 1992).

The 33-kDa catalytic subunit of brain cdc2-like kinase is termed cdk5. cdk5 histone H1 kinase activity was only found in brain although cdk5 was expressed in other human tissues. In contrast, the 25-kDa subunit transcript is expressed only in brain (Lew et al., 1994; Tsai et al., 1994). When overexpressed and purified from *E. coli*, p25 can activate cdk5 *in vitro* . Since further purification of *in vitro* reconstituted cdk5/p25 kinase resulted in an enzyme preparation whose specific activity was similar to that of neuronal cdc2-like kinase purified from brain, this result suggests that, unlike the case with other cyclin-dependent kinases, full activation of cdk5 occurs upon binding of p25 alone without the requirement for exogenous phosphorylation (Lew et al., 1994). However, since Ser 159 in cdk5 is the equivalent position of Thr 161 in $p34^{cdc2}$ and Thr 160 in $p33^{cdk2}$, it was predicted that phosphorylation of cdk5 on Ser 159 might positively regulate cdk5 protein kinase activity.

This proposal was investigated by the activation and phosphorylation of cdk5/p25 by bovine brain cdk activating kinase,

CAK. Bovine brain CAK failed to further activate MONO S purified reconstituted cdk5/p25 kinase in the present work. Moreover, brain CAK failed to phosphorylate cdk5/p25 on cdk5. Therefore, full activation of brain cdk5 only required association with p25 without any requirement for phosphorylation on Ser 159 by brain CAK. In another word, brain cdk5 is not a substrate of brain CAK *in vitro*. However, one can not rule out the possibility of the involvement of other proteins in the cdk5 activation process *in vivo*, such as brain CAK.

Thus brain CAK appears to have very limited substrate specificity. Brain CAK has no activity towards conventional $p34^{cdc2}$ substrates such as histone H1. Except cdk2 as its substrate, if cdk5 is not brain CAK's substrate, it is unclear that how many other cdks brain CAK can phosphorylate. Most likely $p34^{cdc2}$ may be brain CAK's good substrate because $p34^{cdc2}$ is the most similar to cdk2 especially within the region surrounding Thr160 in cdk2. Besides, CAK in Xenopus, Starfish and human have shown the ability of phosphorylation of $p34^{cdc2}$ on Thr 161 (Fisquet et al., 1993; Solomon et al., 1993; Poon et al., 1993; Tassan et al., 1994; Fisher et al., 1994).

Unlike other cdks, brain CAK does not seem to recognize primary sequence determinants alone. First, no peptide substrates have been found for CAK. Second, Thr 160 of cdk2 is found at the apex of a loop

(the 'T-loop') that somehow blocks substrate binding and that points towards the ATP-binding region of the enzyme (De Bondt et al., 1993). It is possible that primary sequence determinants will play only a minor role in recognition by CAK, and that protein kinases sharing little apparent similarity to cdks may also be excellent substrates for CAK (Solomon, 1994). Further search for the substrates of brain CAK both *in vivo* and *in vitro* will be carried out when a highly purified enzyme is obtained. This will enable one to understand the details of regulatory pathways during the cell cycle.

4. Regulation of Brain CAK

Since monomeric $p40^{MO15}$ is not active, its association with a regulatory subunit is essential for CAK activity. The identification of cyclin H and the finding that purified bacterially expressed cyclin H can activate $p40^{MO15}$ in insect cell lysates confirmed this prediction (Fisher et al., 1994). In addition to cyclin H binding, phosphorylation of Thr 176 is required for the activation of $p40^{MO15}$. This site is equivalent to Thr 161 in $p34^{cdc2}$ and Thr 160 in $p33^{cdk2}$. Mutation of Thr 176 to alanine severely reduces subsequent CAK activity (Fisher et al., 1994). Since incubation of recombinant $p40^{MO15}$ in Xenopus egg extracts leads to its activation, but only if ATP is present, $p40^{MO15}$ may require an activating phosphorylation (Poon et al., 1993). The protein kinase which phosphorylates $p40^{MO15}$

on Thr 176 is unknown but has been termed CAK-activating kinase, CAKAK. Completely purifying brain CAK and using purified protein sequence information to clone brain CAK from cDNA library will make it possible to study the upstream regulation of brain CAK. This study will be a focal point for the regulation pattern in terminally differentiated cells. Furthermore, studies on the function of brain CAK will be the first example of a CAK activity in neurons and provide further evidence for the idea that members of cdc family are involved in processes other than cell-cycle control (Lew et al., 1994)

5. Substrate Specificity of cdk2/cyclin A Kinase

The highly purified active reconstituted cdk2/cyclin A kinase enables one to address the possibility that it may posess differences in its substrate specificity determinants. Synthetic peptides were used previously as effective tools to confirm brain cdc2-like kinase (nclk) specific determinants in our lab (Beaudette et al, 1993). Consensus motifs for protein kinases allow the identification of additional unknown substrates and provide important information for the design of synthetic substrates that are specific for the enzyme of interest. In addition, information obtained from detailed kinetic analysis of synthetic peptide substrates may prove useful for the design of potential inhibitory peptides.

The consensus sequence for $p34^{cdc2}$ recognition was proposed based on the analysis of *in vivo* and *in vitro* phosphorylation sites of numerous $p34^{cdc2}$ substrates (Shenoy et al., 1989; Moreno and Nurse, 1990). Using kinetic analysis of histone peptide analogoues, based on *in vivo* $p34^{cdc2}$ phosphorylation sites, the S/T-P-X-K/R motif has essentially been confirmed for the brain nclk (Beaudette et al., 1993).

In the present study, kinetic characterization of synthetic histone peptides has confirmed the importance of $p34^{cdc2}$ consensus sequence residues and revealed additional secondary determinants outside of this motif for cdk2/cyclin A kinase. The primary determinant, a proline at the +1 position, is indispensable as a substrate determinant, as replacement with an alanine residue abolishes its substrate activity. The basic residue at the +3 position exerts a strong positive influence on substrate activity, particularly in binding affinity, as replacement with an alanine residue results in a 74-fold increase in Km value. Finally, the threonine residue is preferred phospho-acceptor as a histone peptide having alanine substituted for threonine is not a substrate for cdk2/cyclin A kinase.

The use of synthetic peptides to elucidate specificity determinants for protein kinases also allows for the investigation

of secondary determinants, those which improve substrate activity but are not essential. In this study, kinetic characterization of synthetic histone peptides has revealed several secondary determinants for cdk2/cyclin A. At the +2 position, a basic residue is positive determinant, while an acidic residue at the same a position is negative determinant for phosphorylation by cdk2/cyclin A. Unlike brain nclk, the proline at the -2 position and lysines at +5 and +6 positions do not appear to play significant roles in the substrate activity of the peptide for cdk2/cyclin A.

cdk2/cyclin A

bovine brain nclk

The present work represents the first attempt to investigate the substrate determinants of a purified reconstituted cdk family member, cdk2/cyclin A kinase, using synthetic peptides. The data

presented in this study demonstrate that both cdk2/cyclin A kinase and brain cdc2-like kinase, nclk, have similar phosphorylation site specificity in that both kinases recognize the consensus sequence motif: S/T-P-X-K-K/R-. However, some of the secondary positive substrate determinants for nclk, such as the proline at -2 and lysines at +5 and +6 are not very important for substrate activity of the peptide for cdk2/cyclin A. This is the first evidence that cdk family members share similar consensus motif even with different cyclin subunits.

The expression, reconstitution and characterization of cdk2/cyclin A kinase has been established in the present study. This system can be used for the study of other *in vitro* associated forms of cdk family members and cyclin family members. With the existence of multiple forms of cdk kinases and cyclins, the set of substrate peptides prepared in this study may be useful in defining possible differences in substrate specificity for these various forms.

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